UNIVERSIDADE DE LISBOA · FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA VEGETAL

CONTRIBUTION OF THE *GJB2* GENE TO NONSYNDROMIC SENSORINEURAL HEARING LOSS IN THE PORTUGUESE POPULATION

Tiago Daniel Lopes Morim Pereira de Matos

DOUTORAMENTO EM BIOLOGIA
(Especialidade Genética)

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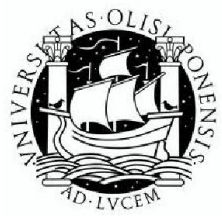

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This thesis was supervised by Professor Doutora Maria da Graça Monteiro de Azevedo Fialho (Center for Biodiversity, Functional and Integrative Genomics, Faculty of Science, University of Lisbon) and by Professor David Kelsell PhD (Center for Cutaneous Research, Barts and The London School of Medicine and Dentistry, Queen Mary, University of London).

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DECLARAÇÃO

Para efeitos do disposto no n°2 do artigo 8 do Decreto-Lei 388/70, declaro que a presente dissertação inclui artigos científicos dos quais sou co-autor.

Tiago Daniel Matos

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List of abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BC	Basal cell
bp	Base pair
cAMP	Cyclic adenosine monophosphate
CDE	Constitutive decay element
CFP	Cyan fluorescent protein
CMTX	X-linked Charcot-Marie-Tooth disease
Cx26	Connexin-26
Cx30	Connexin-30
Cx31	Connexin-31
Cx32	Connexin-32
Cx46	Connexin-46
Cx47	Connexin-47
dB	Decibel
E1/E2	Extracellular loop 1 or 2
EGFP	Enhanced green fluorescent protein
EtBr	Ethidium bromide
FACS	Fluorescence activated cell sorting
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GJA3	Gap junction alpha-3
GJB1	Gap junction beta-1
GJB2	Gap junction beta-2
GJB3	Gap junction beta-3
GJB6	Gap junction beta-6
GJC2	Gap junction gamma-2
HL	Hearing loss

Hz	Hertz
IC	Intermediate cell
IHC	Inner hair cell
IP3	Inositol 1,4,5-trisphosphate
IRE	Iron responsive element
IRES	Internal ribosome entry site
IS	Intrastrial space
ISO	International Organization for Standardization
kbp	Kilobase pair
KCNE1	Potassium voltage-gated channel, subfamily E, member 1
KCNJ10	Potassium inwardly-rectifying channel, subfamily J, member 10
KCNQ1	Potassium voltage-gated channel, subfamily Q, member 1
kHz	Kilohertz
LD	Linkage disequilibrium
LY	Lucifer yellow
M1-M4	Transmembrane domains 1 to 4
MC	Marginal cell
µg	Microgram
MGF	Mammary growth factor
µL	Microliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
mV	Millivolt
NBN	Neurobiotin
NF-kB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
ng	Nanogram
NKCC	Na-K-Cl cotransporter
NSHL	Nonsyndromic hearing loss

NSSHL	Nonsyndromic sensorineural hearing loss
OHC	Outer hair cell
pb	pares de base (portuguese for base pairs)
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
RFLP	Restriction fragment length polymorphism
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SECIS	Selenocysteine insertion sequence
siRNA	Small interfering RNA
SLDE	Stem-loop destabilizing element
SOX10	SRY (sex determining region Y)-box 10
Sp1/3	Specificity protein 1 or 3
SSCP	Single-strand conformation polymorphism
TFRC	Transferrin receptor
TIA-1	T-cell-restricted intracellular antigen-1
TNF-alpha	Tumor necrosis factor alpha
TSP	Transcription start point
V	Volt
Wt	Wild-type
UTR	Untranslated region

Statement of work

The experimental work described in this PhD thesis was developed under the supervision of Prof. Doutora Maria da Graça Monteiro de Azevedo Fialho and Prof. David P. Kelsell PhD, at the Centro de Genética e Biologia Molecular (presently Center for Biodiversity, Functional and Integrative Genomics), Faculty of Science, University of Lisbon, and at the Center for Cutaneous Research, Barts and The London School of Medicine and Dentistry, Queen Mary, University of London, between October 2004 and June 2009.

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List of manuscripts

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Caria, H., **Matos, T.**, Oliveira-Soares, R., Santos, A. R., Galhardo, I., Soares-Almeida, L., Dias, O., *et al*. (2005). A7445G mtDNA mutation present in a Portuguese family exhibiting hereditary deafness and palmoplantar keratoderma. *Journal of the European Academy of Dermatology and Venereology*, *19*(4), 455-458.

Resumo

Mutações no gene *GJB2* são responsáveis por uma percentagem significativa de casos de surdez neurossensorial não sindrómica em várias populações. Este gene pertence à família de genes que codificam as conexinas, as subunidades dos hemi-canais (conexões) que formam os canais intercelulares das *gap junctions* existentes nos vertebrados e nos tunicados. A conexina-26 (Cx26), codificada pelo gene *GJB2*, é expressa em vários tecidos, incluíndo tecidos específicos epiteliais e conjuntivos da cóclea, órgão auditivo localizado no ouvido interno. Na cóclea, a Cx26 é co-expressa com a conexina-30 (Cx30), codificada pelo gene *GJB6*, em vários tipos de células. Duas extensas deleções afectando o gene *GJB6* são também responsáveis por vários casos de surdez, sendo a maior parte deles devido a heterozigotia composta com uma mutação em *GJB2*.

Dada a relevância dos genes *GJB2* e *GJB6* na etiologia da surdez em várias populações, o diagnóstico molecular de casos de surdez neurossensorial não sindrómica é habitualmente iniciado pela análise da região codificante do gene *GJB2* (a maior parte das mutações patogénicas foram encontradas nesta região), seguida da investigação da presença das deleções do gene *GJB6* quando nenhuma ou apenas uma mutação é encontrada no gene *GJB2*. Em certos casos, a atribuição da causa da surdez ao genótipo *GJB2*/*GJB6* é complicada pelo facto de a patogenicidade de algumas mutações do gene *GJB2* ser indeterminada ou controversa, quer por falta de evidência genética (segregação evidente da mutação com a surdez), quer devido a dados genéticos contraditórios (indivíduos com audição normal e indivíduos com surdez que apresentam o mesmo genótipo). Nesses casos, os estudos funcionais constituem um meio adequado de investigar a eventual patogenicidade dessas mutações, tendo várias sido já desde modo analisadas.

Um dos primeiros objectivos deste trabalho foi o estudo da região codificante e local de *splicing* receptor do gene *GJB2* em pacientes portugueses com surdez neurossensorial não sindrómica. Estendemos, seguidamente, a análise do gene a regiões não codificantes (promotor basal e os cerca de 700 pb imediatamente a montante, o exão 1, o local de splicing dador e toda a 3'UTR). Estas regiões não codificantes têm sido até agora raramente estudadas. No entanto, duas mutações patogénicas não codificantes tinham sido já identificadas, ambas no local dador de splicing. Inicialmente, analisámos apenas alguns indivíduos portadores de uma mutação

codificante, relativamente ao promotor basal, exão 1 e local dador de splicing. No decorrer desta análise inicial de regiões não codificantes, encontrámos uma nova mutação (c.-259 C>T) no promotor basal, num dos locais de ligação de factores de transcrição Sp1/Sp3. Esta mutação foi encontrada numa paciente com surdez profunda, em *trans* com p.Val84Met, por nós identificada anteriormente como uma nova mutação associada a surdez em *GJB2*.

Posteriormente, analisámos todas as regiões não codificantes atrás referidas, num conjunto maior de pacientes (n=89), com apenas uma ou nenhuma mutação codificante em *GJB2*, e em 91 controlos, considerados como tendo audição normal. Os resultados decorrentes deste análise revelaram que, nos pacientes, está sobre-representado o genótipo c.[*168A>G(+)*931C>T], um duplo heterozigótico respeitante aos SNPs rs55704559 e rs5030700 (ambos localizados na 3'UTR), respectivamente. A análise *in silico* prevê que a variante c.*168A>G (independentemente de estar uma citosina ou uma timina na posição c.*931) cause uma alteração da conformação do mRNA. Deste modo, os nossos dados sugerem que o alelo c.*168G poderá estar associado a surdez.

Outro dos objectivos deste trabalho foi a elucidação da patogenicidade das referidas mutações c.-259C>T e p.Val84Met, assim como da mutação p.Met163Leu, também encontrada pela primeira vez, num trabalho prévio, numa outra família Portuguesa. Os dados genéticos disponíveis sobre as três mutações (os nossos e os publicados por outros autores) eram insuficientes para a confirmação da sua patogenicidade, e assim efectuámos estudos funcionais para investigarmos os seus efeitos.

Os resultados obtidos sugerem que as três mutações são de facto patogénicas, exercendo o seu efeito de formas distintas. A mutação c.-259C>T reduz muito significativamente a actividade do promotor basal. Relativamente às mutações codificantes, enquanto a p.Val84Met altera as propriedades de permeabilidade iónica e molecular do canal intercelular, o que poderá *in vivo* traduzir-se num défice funcional, a p.Met163Leu causa a morte celular, possivelmente por outro mecanismo que não o funcionamento anómalo de conexões não emparelhados (já descrito na literatura), e apresenta um efeito dominante negativo parcial sobre a Cx26 e Cx30 do tipo selvagem.

Abstract

Mutations in the *GJB2* gene are responsible for a considerable proportion of nonsyndromic sensorineural hearing loss (NSSHL), in several populations. This gene is a member of a gene family coding for connexins, the subunits of the hemichannels (connexons) which form the intercellular channels of the gap junctions existing in the vertebrates and tunicates. Connexin-26 (Cx26), encoded by the *GJB2* gene, is expressed in several tissues, including specific epithelial and conjunctive tissues of the cochlea, the auditory organ which is localised to the inner ear. In the cochlea, Cx26 is co-expressed with connexin-30 (Cx30), encoded by the *GJB6* gene, in several cell types. Two large *GJB6* deletions are involved in several hearing loss (HL) cases, as well, being most of these cases due to compound heterozygosity with a *GJB2* mutation.

Given the relevance of both *GJB2* and *GJB6* genes to the HL etiology in several populations, the molecular diagnosis of NSSHL cases with probable genetic cause is usually initiated by the analysis of the *GJB2* coding region (in which most pathogenic mutations have been found), followed by the investigation of the presence of the *GJB6* deletions in the cases where none or only one *GJB2* mutation is found. In some cases, attributing the cause of the HL to the *GJB2*/*GJB6* genotype is complicated because some *GJB2* mutations are of unclear or controversial pathogenicity, due to either lack of genetic evidence (evident segregation of the mutation with the HL) or contradictory genetic data (same genotype occurring in both normal-hearing and hearing-impaired individuals). In those cases, functional studies constitute an adequate approach for the investigation of the putative pathogenicity of such mutations, several of which have already been studied in this way.

One of the aims of this work consisted in the study of the coding region and acceptor splice site of the *GJB2* gene in Portuguese individuals presenting with NSSHL. We have further extended the analysis of the gene to noncoding regions (basal promoter and about 700 bp immediately upstream, exon 1, donor splice site and the whole 3'UTR). These noncoding regions have rarely been studied. Nonetheless, two pathogenic noncoding mutations had previously been identified, both in the donor splice site. Initially, we analysed the basal promoter, exon 1 and donor splice site in some patients who only harboured one coding mutation. We then found

a novel mutation (c.-259C>T) in the basal promoter, in one of the binding sites for Sp1/Sp3 transcription factors. This mutation was found in a profoundly hearing-impaired patient, in *trans* with p.Val84Met, that we had identified as a novel mutation, in a previous work.

Later, we analised all the fore mentioned noncoding regions in a larger sample, including monoallelic patients and also those harbouring no mutation in the *GJB2* coding region (n=89) as well as 91 controls who reported to have normal hearing. The obtained data revealed that the c.[*168A>G(+)*931C>T] double heterozygous genotype, regarding the rs55704559 and rs5030700 SNPs (both localised to the 3'UTR), respectively, is overrepresented in the patients. *In silico* analysis predicts that the c.*168A>G variant (regardless of the fact that in position c.*931 is a cytosine or a thymine) causes an alteration of mRNA folding. Thus, our data suggest that the c.*168G allele might be associated with HL.

Other aim of this work was to investigate the effects of the mutations c.-259C>T and p.Val84Met, mentioned above, and the mutation p.Met163Leu, also identified for the first time in a previous work, in other Portuguese family. The available genetic data regarding these mutations were insufficient to prove their pathogenicity, and thus we have performed functional studies on the three mutations. The obtained results suggest that the three mutations are indeed pathogenic, exerting their effect in distinct ways. The c.-259C>T mutation decreases very significantly the basal promoter activity. As regards the coding mutations, p.Val84Met alters the ionic and molecular permeability properties of the intercellular channel, which may compromise their function *in vivo*, while p.Met163Leu leads to cell death, possibly by a mechanism other than the malfunctioning of undocked hemichannels (already described in the literature), and has a partial dominant-negative effect on wild-type Cx26 (wtCx26) and Cx30 (wtCx30).

CHAPTER 1

General Introduction

Section 1 - Physiology of Hearing

The mammalian auditory system is capable of detecting and analyzing sounds over a wide spectrum of frequencies (humans can hear sounds between 20 Hz to 20 kHz) and over an intensity range of 12 orders of magnitude or 120 dB (Robles & Ruggero, 2001).

Ear Anatomy

The human auditory system, responsible for the complex process of hearing, is composed of the outer ear, middle ear and inner ear (fig. 1).

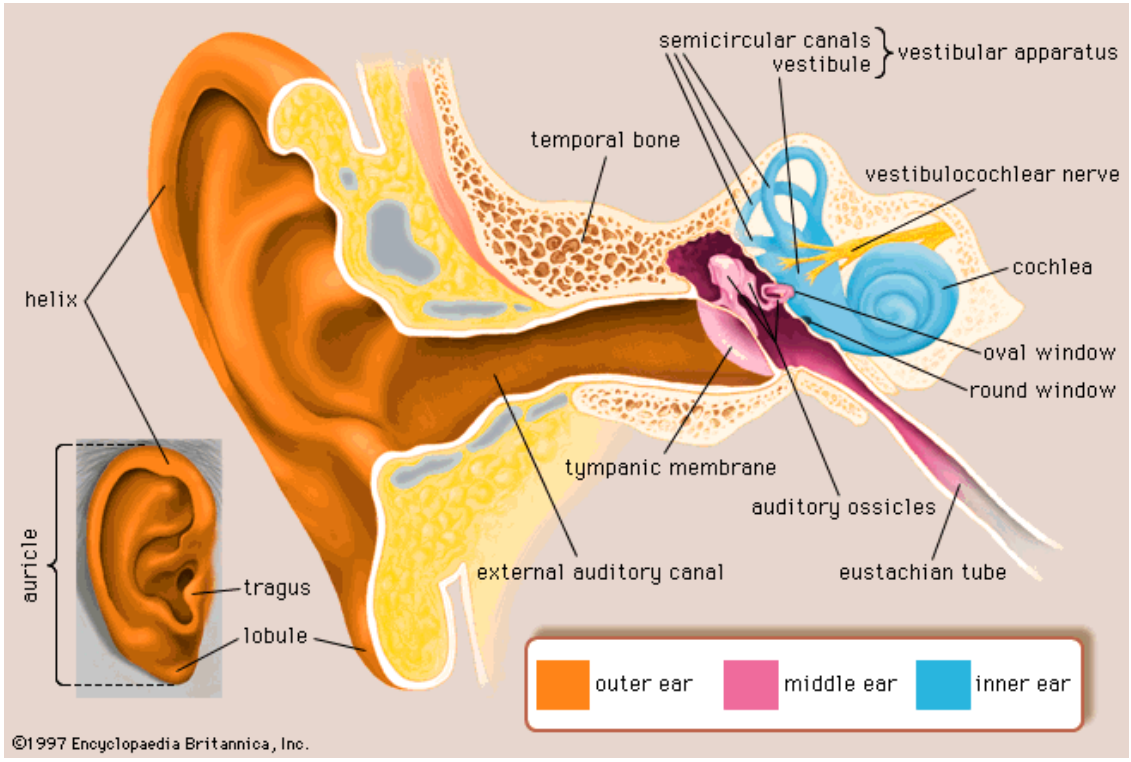

Figure 1. Structure of the human ear. Reproduced from Encyclopædia Britannica Inc. (n.d.).

The outer ear is composed of the external ear (auricle) and the ear canal (external auditory canal). The middle ear includes the tympanic membrane, at the end of the ear canal, and three ossicles (*maleus*, *incus* and *stapes*). The middle ear cavity is connected by the

Eustachian tube to the pharynx. The inner ear consists of the vestibular apparatus, responsible for the balance function, and the cochlea, the organ of hearing (Møller, 2006).

The human cochlea is a structure coiled in the form of a snail (fig. 2), which number of turns is generally considered to be 2 ½. However, a study conducted by (Biedron, Westhofen, & Ilgner, 2009) suggests that the majority of individuals (65% in their study) has more than 2 ½ cochlear turns, of which a small fraction has more than 2 ¾ turns. The cochlea is composed of three contiguous, fluid-filled, membranous tubes, *scala vestibuli*, *scala tympani* and *scala media* (fig. 2), being enclosed by a bony shell, the otic capsule.

The *scala vestibuli* and *scala tympani*, which communicate near the apex of the otic capsule, at helicotrema (fig. 2), are filled with perilymph, a fluid with an ionic composition similar to that of other extracellular fluids. The *scala media* is filled with endolymph, a fluid that, contrary to perilymph, has a high potassium concentration (Wangemann, 2006), which is necessary for the normal auditory function.

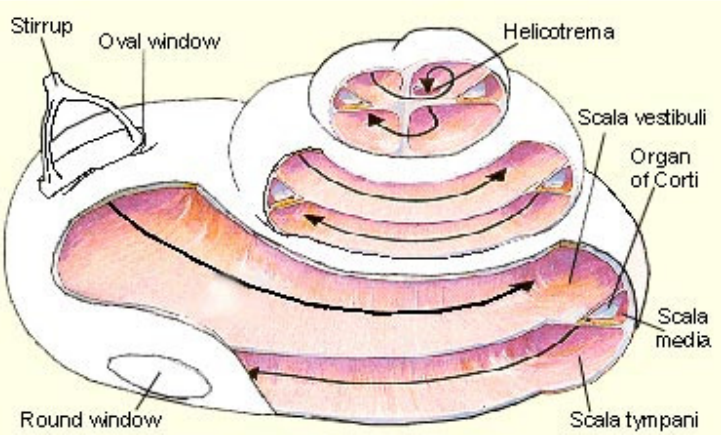

Figure 2. Cochlea opened from the side. The three compartments (*scala vestibuli*, *scala media*, *scala tympani*) are shown. The arrows indicate the direction of the sound wave traveling through the perilymph along *scala vestibuli* and *scala tympani*, which are connected at helicotrema. The stirrup is also called the stapes. Figure reproduced from Hearing Central LLC (n.d.).

Transduction of sound into nerve impulses

The sound is conducted through the outer ear to the tympanic membrane, which transmits vibration to the three ossicles of the middle ear. These ossicles impart the vibration to the oval window of the cochlea (fig. 2), creating a mechanical wave in the cochlear fluid and in the basilar membrane (fig. 3). The mechanical vibration is transduced into electric signals in the organ of Corti (fig. 3), which contains several cell types, including inner and outer hair cells (IHCs and OHCs, respectively) as well as supporting cells (e.g. inner and outer pillar cells, Claudius', Hensen's and Deiters' cells) (fig. 3).

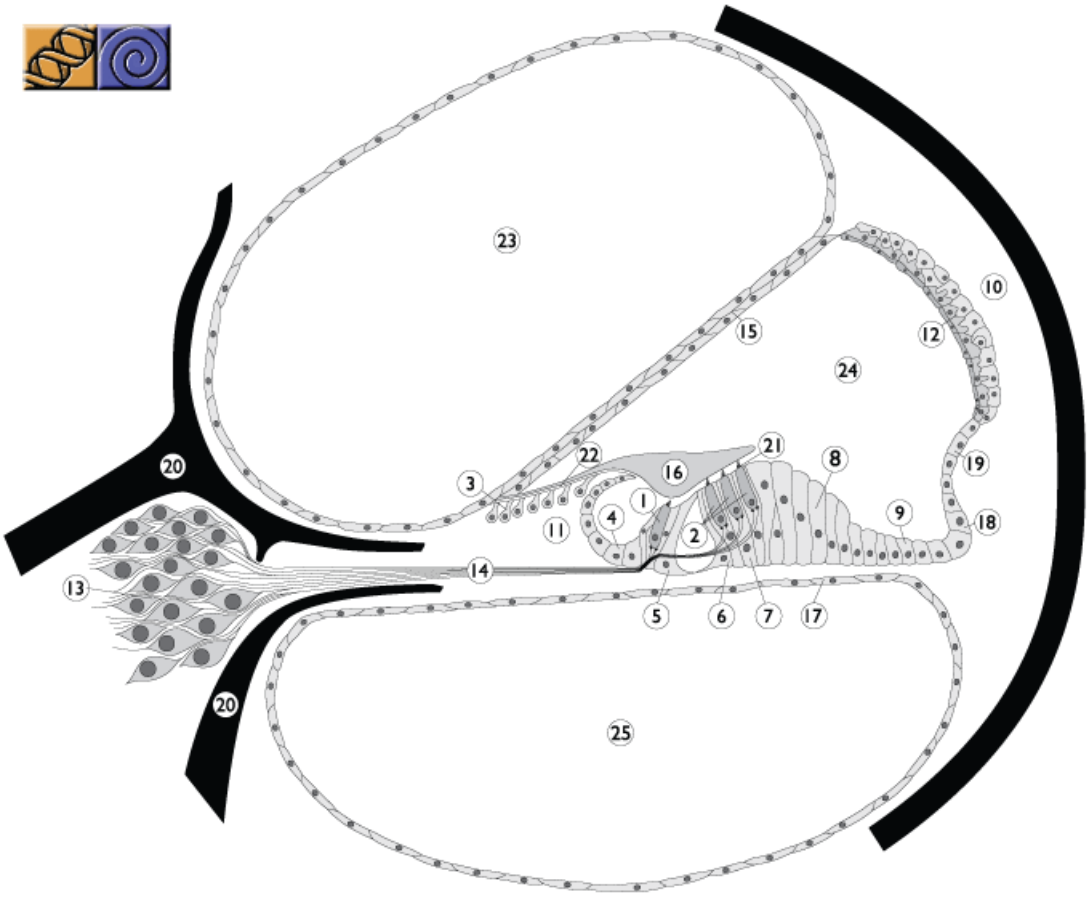

Figure 3. Representation of a cross-section of one turn of the cochlea, showing the three compartments, *scala vestibuli*, *scala media* and *scala tympani*, and the organ of Corti, resting on the basilar membrane. 1. Inner hair cell; 2. Outer hair cell; 3. Interdental cell (IDC); 4. Inner sulcus cells; 5. Inner pillar cells; 6. Outer pillar cells; 7. Deiters' cells; 8. Hensen's cells; 9. Claudius' cells; 10. Spiral ligament; 11. Spiral limbus; 12. *Stria vascularis*; 13. Spiral ganglion; 14. Auditory nerve; 15. Reissner's membrane; 16. Tectorial membrane (TM); 17. Basilar membrane; 18. External sulcus cells; 19. Spiral prominence; 20. Bony spiral lamina; 21. Reticular lamina; 22. Between IDC and TM; 23. *Scala vestibuli*; 24. *Scala media*; 25. *Scala tympani*. Reproduced from Van Camp & Smith (2011a).

The OHCs play an active role in increasing sensitivity to low intensity acoustic stimuli, and in frequency selectivity (especially for weak sounds) while the IHCs are responsible for the transduction of mechanical vibration (caused by sound) into nerve impulses (Møller, 2006). The movement of the basilar membrane leads to the deflection of the stereocilia at the apical

surface of IHCs and OHCs, which elicits the opening of the transducer channels (Beurg, Evans, Hackney, & Fettiplace, 2006; Ricci, Kennedy, Crawford, & Fettiplace, 2005; Stauffer & Holt, 2007). The current that then passes through these non-selective cation channels, carried by Ca^{2+} and mainly by K^{+} (LeMasurier & Gillespie, 2005), leads to depolarization of the cell's membrane. Influx of calcium ions through IHCs' voltage-gated Ca^{2+} channels elicits exocytosis (Brandt, Striessnig, & Moser, 2003; Moser & Beutner, 2000) and consequently the release of the neurotransmitter which stimulates type I afferent fibers via glutamatergic synapses, involving the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and the glutamate-aspartate transporter (GLAST) (Fuchs, Glowatzki, & Moser, 2003; Glowatzki *et al*., 2006). This primary acoustic input is then transmitted to the brain.

K^{+} recycling

The process of transduction requires K^{+} to be returned to the endolymph. It is believed that K^{+} ions that exit hair cells through K^{+} channels at their basolateral membrane into the perilymph may be taken up by supporting cells (Hibino & Kurachi, 2006; Zdebik, Wangemann, & Jentsch, 2009), and move either through supporting cells, epithelial cells, and fibrocytes of the spiral limbus back into the endolymph, or through supporting and epithelial cells to the spiral ligament, where they are taken up by fibrocytes and conducted via the fibrocyte network to the *stria vascularis* where they are then secreted back into the endolymph (Spicer & Schulte, 1998) (fig. 4).

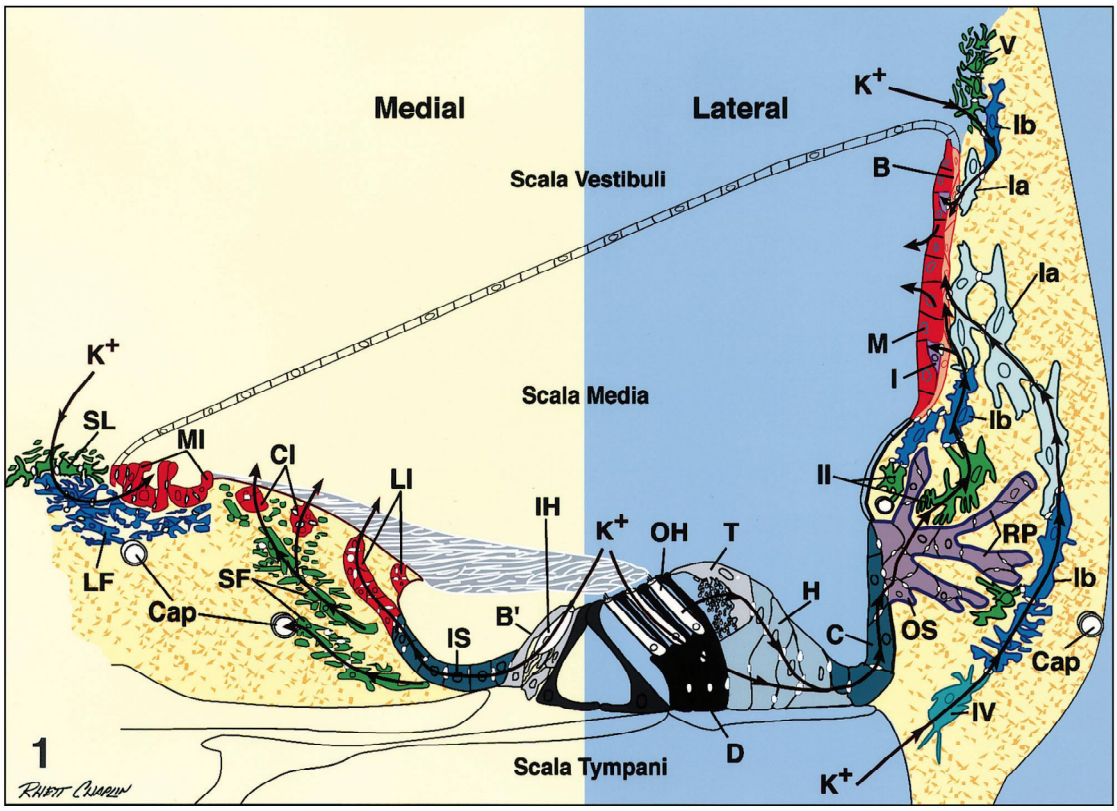

Figure 4. A schematic representation of the proposed transcellular pathways of K^+ ions, effluxed from hair cells during auditory transduction, back to the endolymph. B=basal cell; BP=border cell; Cap=capillary; C=Claudius' cell; CI=central interdental cell; D=Deiters' cell; H=Hensen's cell; I=intermediate cell; IH=inner hair cell; IS=inner sulcus cell; M=marginal cell; MI=medial interdental cell; LF =light fybrocyte; LI=lateral interdental cell; OH=outer hair cell; OS=outer sulcus cell; RP=root process; SF =stellate fybrocyte; SL=supralimbal fybrocyte; T=tectal cell; Ia, Ib, II, IV, and V=types of lateral wall fybrocytes. Reproduced from Spicer and Schulte (1998).

In addition to K^+ circulation induced by acoustic stimulation, evidence supports the existence of standing currents in the cochlea (fig. 5), not depending on acoustic stimulation but being modulated by it (Zidanic & Brownell, 1990).

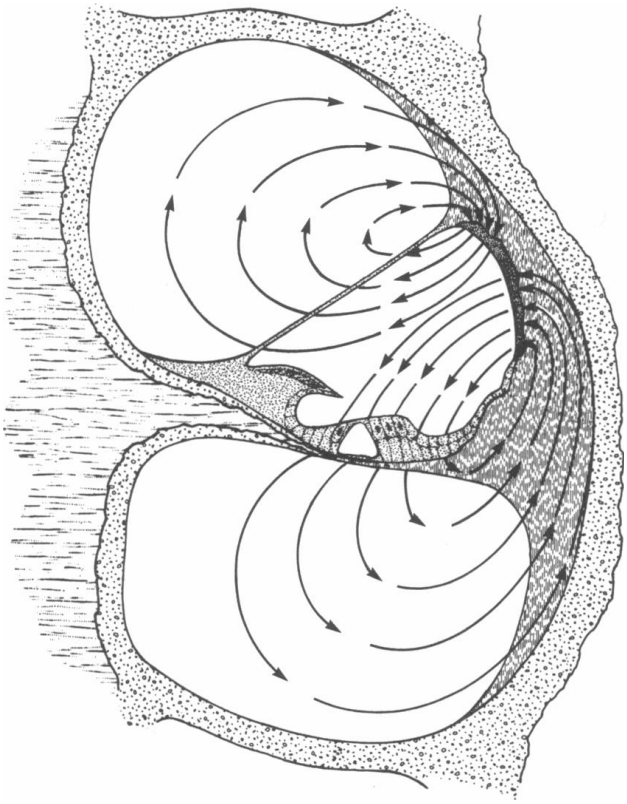

Figure 5. Model for standing currents in the cochlea in terms of current density field lines. The current leaks from *scala media* through the mechanically-sensitive transduction channels in the stereocilia of hair cells. Current leakage from *scala media* is thought to occur also through Reissner's membrane and through (or between) the supporting cells lateral to the organ of Corti. Reproduced from Zidanic and Brownell (1990).

Stria vascularis and the endocochlear potential (EP)

The *stria vascularis*, located in the cochlear lateral wall, is a tissue composed of two epithelial layers, with one composed of basal cells (BCs) and intermediate cells (ICs), and the other composed of marginal cells (MCs). The space that exists between the MCs and ICs is designated by intrastrial space (IS), and contains a dense capillary network (fig. 6).

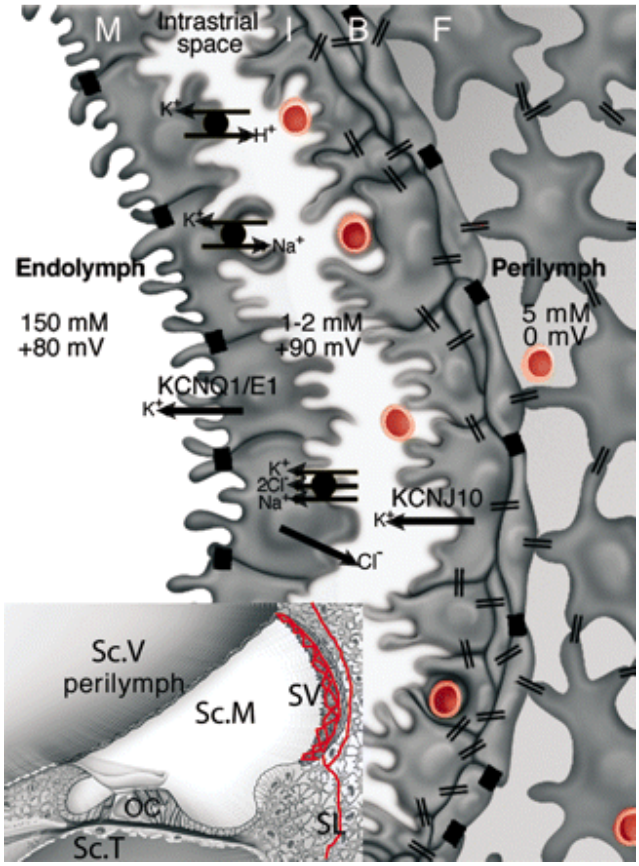

Figure 6. Schematic diagrams illustrating the structure and location in the cochlea of the *stria vascularis* and the electrogenic machinery involved in the endocochlear potential generation. Inset: cross section of a cochlea's turn. OC = organ of Corti; Sc.V = *scala vestibuli*; Sc.M = *scala media*; Sc.T = *scala tympani*; SL = spiral ligament; SV = *stria vascularis*. Red lines represent the vascular system of the cochlea lateral wall. Large diagram: *stria vascularis* in detail. B = basal cells; I = intermediate cells; M = marginal cells. Black boxes indicate tight junctions (TJs) between marginal cells and between basal cells. Endothelial cells of the SV capillaries are also linked by TJs (not represented). Intermediate and basal cells of the SV and fibrocytes (F) of the SL are connected by gap junctions (GJs) (double bars) composed of Cx26 and Cx30 (Forge *et al*., 2003; Lautermann *et al*., 1998; W. Liu, Boström, Kinnefors, & Rask-Andersen, 2009; Y.-P. Liu & Zhao, 2008). The K^+ concentration (in mM) and the electric potential (in mV) are indicated in each of the three extracellular fluid spaces, namely the perilymph, intrastrial fluid, and endolymph. Each electric potential is indicated relative to that of the perilymph, which is taken as reference (0 mV). Figure reproduced from Cohen-Salmon *et al*. (2007).

As mentioned previously, *stria vascularis* secretes K^+ ions into the endolymph. Additionally, *stria vascularis* generates an EP of approximately +80 mV which constitutes the main driving force for sensory transduction (Wangemann, 2002), thus being critical for the normal auditory function. The EP comprises two different K^+-diffusion potentials, one across the apical membrane of the ICs and the other across the apical side of the MCs (Nin *et al*., 2008). This EP is dependent on KCNJ10 (Kir4.1) (Marcus, Wu, Wangemann, & Kofuji, 2002; Nin *et al*., 2008) and KCNQ1/KCNE1 (Nin *et al*., 2008) K^+ channels, localised to the apical surfaces of ICs and MCs, respectively. The Na^+,K^+-ATPase and NKCC K^+ transporters, that exist in the basolateral membrane of MCs, are also important for the generation of the EP (Nin *et al*., 2008). The gastric H^+,K^+-ATPase is expressed at the basolateral membrane of MCs as well, and may participate in EP formation (Shibata *et al*., 2006), but its role in the regulation of the electrochemical milieu of the IS remains unclear (Nin *et al*., 2008).

Cochlear gap junction systems

Two gap junction systems, the epithelial cell and the connective cell gap junction systems, exist in the cochlea and are thought to play a role in the recirculation of K^+ by providing a mechanism for the uptake of the K^+ released by the hair cells, its transcellular transport laterally, and its delivery to the *stria vascularis* (Kikuchi, Adams, Miyabe, So, & Kobayashi, 2000; Kikuchi, Kimura, Paul, & Adams, 1995; Kikuchi, Kimura, Paul, Takasaka, & Adams, 2000). Spicer and Schulte (1998) suggested the existence of a medial K^+ recycling pathway from IHCs, in parallel with the formerly hypothesized lateral pathway (fig. 4), implicating the participation of gap junctions in both transcellular routes. Jagger and Forge (2006) presented evidence for the existence of two distinct compartments (medial and lateral) in the organ of Corti, which are isolated from each other, in terms of gap junctional intercellular communication. This finding supports Spicer and Schulte's model of two distinct transcellular routes (medial and lateral) for the recirculation of K^+, in which gap junctions would play a role, but it also demonstrated the existence of gap junction channels with different permeability properties, suggesting that the gap junction intercellular communication may play additional roles in the organ of Corti beyond its putative contribution for K^+ recycling.

Section 2 - Hearing loss

Hearing loss is the most common sensorial impairment being a major public health concern. In 2005, about 278 million people had moderate to profound hearing impairment, of whom 80% live in low- and middle-income countries (World Health Organization, 2011a). HL affects 6 to 8% of the population in developed nations (Schrijver, 2004). This condition can occur at any age. In children before speech, HL (prelingual HL) occurs in 1 in 800-1000 while in early childhood HL occurs in 1 in 400-500. The probability of impairment increases with age (phenomenon designated by age-related HL or presbyacusis), affecting 2.3 % of the population aged 40-50 years, and over 30% of the population above 70 years of age (Petit *et al*., n.d.). The auditory defect can be located in the external or middle ear (conductive HL), or in the inner ear (sensorineural HL), affecting the cochlea or the auditory nervous system. Mixed HL results from anomalies in both external/middle ear and inner ear. HL may present itself with variable grades, which are described in table 1.

Table 1. Grades of hearing impairment, adapted from World Health Organization (2011b). Grades 2, 3 and 4 are classified as disabling hearing impairment. The audiometric ISO values are averages of values at 500, 1000, 2000, 4000 Hz.

Grade of impairment	Corresponding audiometric ISO value	Performance
0 - None	25 dB or better (better ear)	No or very slight hearing problems. Able to hear whispers.
1 - Slight (Mild)	26-40 dB (better ear)	Able to hear and repeat words spoken in normal voice at 1 metre.
2 - Moderate	41-60 dB (better ear)	Able to hear and repeat words spoken in raised voice at 1 metre.
3 - Severe	61-80 dB (better ear)	Able to hear some words when shouted into better ear.
4 - Profound	81 dB or greater (better ear)	Unable to hear and understand even a shouted voice.

HL can be caused by environmental factors (infections, prematurity, trauma and exposition to ototoxic medications), however, in developed countries, most cases have a genetic cause.

Section 3 - Genetic hearing loss

Genetic hearing loss can be syndromic (~30%) or nonsyndromic (~70%). About 80% of nonsyndromic hearing loss (NSHL) cases are autosomal recessive, 15-20% are autosomal dominant, about 1% are X-linked and at least 1% are due to mutations in the mitochondrial DNA (mtDNA) (Schrijver, 2004).

To date 60 nuclear genes associated with NSHL have been cloned (Van Camp & Smith, 2011b). While eight of them are associated with both autosomal recessive and dominant HL, 17 genes have been strictly associated with autosomal dominant HL, and 32 other genes are so far related with autosomal recessive HL only. Three genes are implicated in X-linked NSHL. Two mitochondrial genes are also associated with NSHL.

Mutations in one particular gene, *GJB2*, coding for the Cx26 protein, are a major cause of NSHL/NSSHL in several populations (Kenneson, Van Naarden Braun, & Boyle, 2002). As such, screening for mutations in this gene is generally the first step undertaken in the molecular diagnosis of this pathology. Two gross deletions involving one other connexin gene, *GJB6* (Cx30) (F. J. del Castillo *et al*., 2005; I. del Castillo *et al*., 2002; Lerer *et al*., 2001; Pallares-Ruiz, Blanchet, Mondain, Claustres, & Roux, 2002) have also been associated with NSHL in several cases.

Section 4 - The role of *GJB2* (Cx26) and *GJB6* (Cx30) genes in hearing loss

The Connexins and Gap Junctions

Connexins are transmembrane proteins, with four transmembrane domains (M1-M4), two extracellular loops (E1, E2), a cytoplasmic loop and cytoplasmic amino (N)- and carboxy (C)-termini (Kumar & Gilula, 1996). The N-terminus, transmembrane domains and extracellular loops are highly conserved among connexins, while the cytoplasmic loop and C-terminus are quite variable in both length and sequence (Haefliger *et al*., 1992).

Connexins oligomerize into hemichannels termed connexons, which are assembled from six connexin subunits (Baker, Caspar, Hollingshead, & Goodenough, 1983; Makowski, Caspar,

Phillips, & Goodenough, 1977; Perkins, Goodenough, & Sosinsky, 1997; Unwin & Zampighi, 1980; Yeager, 1998). Two connexons in adjacent plasma membranes dock forming an intercellular channel (fig. 7).

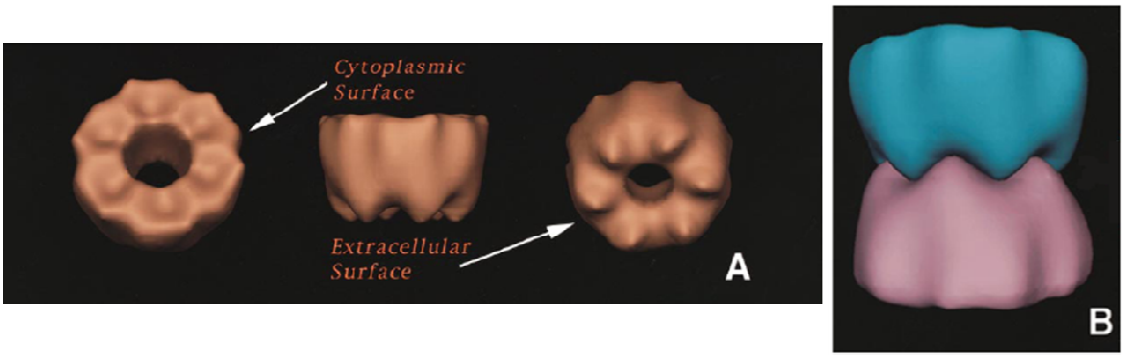

Figure 7. Tri-dimensional representation of a connexon (A), and of two connexons docked to form a intercellular channel (B). Figure adapted from Perkins, Goodenough, and Sosinsky (1998).

Several of these intercellular channels cluster into structures termed gap junctions. Channels formed by connexins have been shown to be permeable to atomic ions, ADP, ATP, glutathione, glutamate (Harris, 2001), second messengers such as cAMP and IP3 (Harris, 2007), and even siRNAs (Valiunas *et al*., 2005). Connexins are expressed in several tissues (e.g. skin, nervous system, respiratory epithelium, bone, lens, heart and vasculature, reproductive system, inner ear) (Harris & Locke, 2009), playing key roles in tissue physiology. For example, these proteins are of importance in keratinocyte biology, being involved in skin differentiation and in wound healing (Aasen & Kelsell, 2009; Langlois *et al*., 2007; C. M. Wang, Lincoln, Cook, & Becker, 2007). Connexins are also involved in the electric conduction in myocardium (Kanno & Saffitz, 2001), and in the electric signaling between neurons (Connors, 2009), and contribute to tissue homeostasis (Mathias, White, & Gong, 2010). Connexons can be assembled from six subunits of the same connexin (homomeric connexons) or from different connexins (heteromeric connexons). The intercellular channels are homotypic if both connexons are identical, or heterotypic if they are composed of two different connexons. Heteromeric

connexons have been found in several tissues (Diez, Ahmad, & Evans, 1999; He, Jiang, Taffet, & Burt, 1999; Jiang & Goodenough, 1996; Locke *et al*., 2007, 2000), including cochlear tissues (Shoab Ahmad, Chen, Sun, & Lin, 2003; Forge *et al*., 2003; X. Z. Liu, Yuan, *et al*., 2009).

Genomic data has provided evidence of the generalised presence of connexins in vertebrates (Cruciani & Mikalsen, 2006). Connexins have also been identified in tunicates (Sasakura *et al*., 2003; Seo *et al*., 2001; White, Wang, Mui, Litteral, & Brink, 2004). These findings, together with additional genomic data from other taxonomic groups obtained so far, suggest that this protein family may be specific to vertebrates and tunicates (Panchin, 2005; Shestopalov & Panchin, 2008; Sodergren *et al*., 2006).

The GJB2 and GJB6 genes (DFNB1/DFNA3 loci)

Twenty connexin genes have been identified in humans (Cruciani & Mikalsen, 2005). The genes *GJB2* and *GJB6*, together with gene *GJA3*, which codes for connexin-46 (Cx46), form a connexin gene cluster which localise to chromosome 13q12.11 (fig. 8).

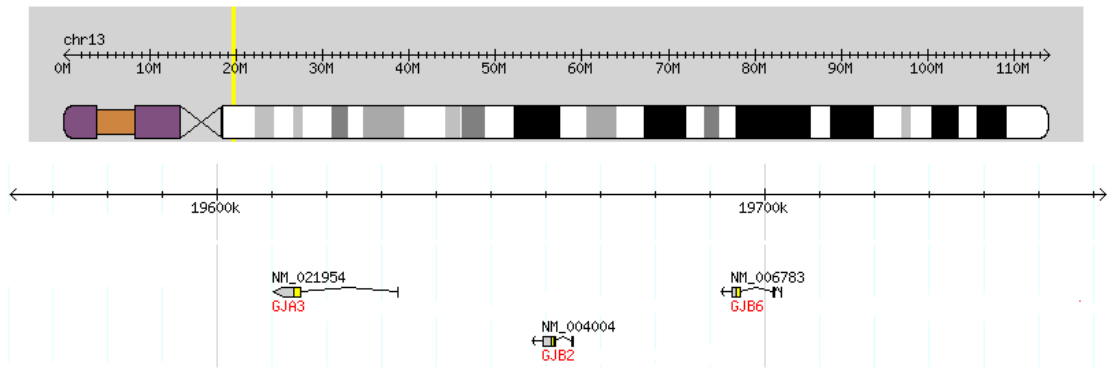

Figure 8. *GJA3*, *GJB2* and *GJB6* connexin gene cluster in human chromosome 13q12.11. Adapted from The International HapMap Consortium (n.d.).

The genomic region comprising the *GJB2* and *GJB6* genes is commonly known as the DFNB1 locus or the DFNA3 locus, when referring to mutations in these connexins causing

recessive or dominant HL, respectively (Smith & Van Camp, 1998; Smith, Sheffield, & Van Camp, 1998).

The *GJB2* gene comprises a first noncoding exon (different TSPs are suggested by distinct 5' capped cDNA sequences), a 3179 kb intron, and a second exon containing the whole coding region (fig. 9a). There is also supporting evidence for an unspliced transcript, containing a shorter ORF initiating at an AUG upstream of the main ORF (fig. 9b), which is likely to impair the efficacy of the translation of the Cx26 protein. Additionally, there are alternative polyadenylation sites (figs. 9a, b). The possibility of alternative splicing, by means of the splicing out of a 2357 pb intron (fig. 9c), is supported by one cDNA sequence (D. Thierry-Mieg & Thierry-Mieg, 2010).

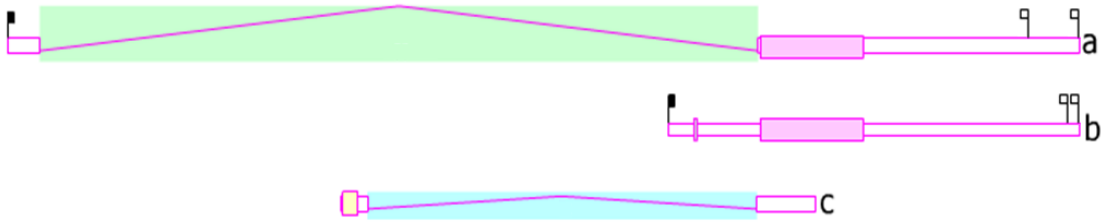

Figure 9. Scheme of human *GJB2* alternative transcripts. a and c represent spliced transcripts while b represents an unspliced transcript. Filled flags represent 5' cap sites; empty flags represent validated polyadenylation sites (three different sites). Adapted from AceView (D. Thierry-Mieg & Thierry-Mieg, 2010).

In the *GJB6* gene the coding region is contained within a single exon, preceded of five upstream noncoding exons (Essenfelder, Larderet, Waksman, & Lamartine, 2005).

Cx26 and Cx30 in the cochlea

Cx26 and Cx30 are expressed in rodent (Forge *et al*., 2003) and human (W. Liu, Boström, *et al*., 2009) cochleae, in the supporting cells of the organ of Corti and in the lateral wall, where they are part of the two independent gap junction systems, the epithelial cell gap junction

system and the connective tissue cell gap junction system (Kikuchi *et al*., 1995). W. Liu, Boström, *et al*. (2009) also observed expression of the two connexins in human spiral ganglion neurons.

Co-localisation of Cx26 and Cx30 at the same gap junction plaques was observed in supporting cells of the organ of Corti, in basal and intermediate cells of *stria vascularis* and in fibrocytes of the spiral ligament, in rodents (Forge *et al*., 2003). In the same study, an immunoprecipitation assay using mature mouse cochleae demonstrated the existence of heteromeric connexons comprising both Cx26 and Cx30. In humans, co-localisation was detected in the basal cell layer of the *stria vascularis* and in Deiters' cells of the organ of Corti (W. Liu, Boström, *et al*., 2009). Therefore, it is very likely that heteromeric connexons, and/or heterotypic channels, comprising both Cx26 and Cx30 also exist in the human cochlea.

Despite of being co-localised in certain cochlear regions, Cx26 and Cx30 display distinct localisation patterns in the cochlear sensory epithelium (W. Liu, Boström, *et al*., 2009; Zhao & Yu, 2006) and in the cochlear lateral wall (W. Liu, Boström, *et al*., 2009; Y.-P. Liu & Zhao, 2008).

Since Cx26 and Cx30 are constituents of the cochlear gap junction systems, mutations in these genes have been considered to cause HL by interfering with the recirculation of K^+ in the cochlea (Kikuchi, Adams, *et al*., 2000). However, a more complex role of both connexins in cochlear physiology has been emerging in the recent years.

Loss of Cx30 in mice impairs the development of the EP (Teubner *et al*., 2003), through disruption of the endothelial barrier in the *stria vascularis* (Cohen-Salmon *et al*., 2007). Cx30-null mice also present degeneration of the organ of Corti (Teubner *et al*., 2003). Shoeb Ahmad *et al*. (2007) observed that Cx30-null mice express a reduced Cx26 protein level in the cochlea and, interestingly, the consequent hearing impairment is prevented in Cx30-null mice overexpressing Cx26, which present levels of the protein close to the wild-type mice. A recent study has detected a reduction of Cx26 mRNA and protein levels in Cx30-null mice cochlea, particularly significant in cells from the region of the outer sulcus when compared to the whole cochlea. The authors presented evidence of co-regulation of Cx26 and Cx30 through NF-kB pathway as a feature of supporting cells in the outer sulcus region (Ortolano *et al*., 2008). Chang, Tang, Ahmad, Zhou, and Lin (2008) found that Cx30-null mice presented near normal ionic coupling but impaired metabolic coupling in outer sulcus and Claudius' supporting cells,

which normally co-express Cx26 and Cx30, as assessed by Zhao and Yu (2006), in guinea-pig Claudius' cells, and by Y.-P. Liu and Zhao (2008), in rat outer sulcus cells. The results of the fore mentioned studies suggest that ablation of Cx30 and the subsequent decrease of the Cx26 protein levels in supporting cells, results in impaired metabolic coupling, which could explain cell death in the organ of Corti.

Loss of expression of Cx26 in the mice epithelial gap junction network specifically does not seem to impair the EP development, but results in the degeneration of the organ of Corti soon after onset of hearing. It was proposed that the degeneration could be due to local extracellular accumulation of K^+ near the basolateral region of IHCs (Cohen-Salmon *et al*., 2002). However, the fore mentioned finding of the co-regulation of Cx26 and Cx30 could suggest that a downregulation of Cx30 occurs in these conditional Cx26 knocked-out mice, leading to a defective epithelial gap junction system, and consequently to metabolic coupling impairment and cell death.

Additional evidence suggesting the participation of Cx26 and Cx30 in cochlear homeostasis, beyond their putative role in the recirculation of K^+, arises from functional studies of some Cx26 and Cx30 mutant proteins. It was found that some mutations in these connexins (p.Val84Leu, p.Ala88Ser in Cx26, and p.Thr5Met in Cx30) do not impair ionic permeability of intercellular channels but impair their permeability to the larger cationic molecule propidium iodide (PI), as well as to the anionic second messenger IP3, which initiated propagation of Ca^{2+} waves in organotypic cochlear cultures (Beltramello, Piazza, Bukauskas, Pozzan, & Mammano, 2005; Y. Zhang *et al*., 2005). Also, the study of other Cx26 mutants (p.Thr8Met and p.Asn206Ser) showed that mutant and wild-type channels had similar unitary conductance, and that permeability to the anionic molecules cAMP and Lucifer yellow (LY) were retained by the mutant channels whereas their permeability to the cationic dye ethydium bromide (EtBr) was greatly reduced compared with that of the wild-type channel. This finding indicated differential selectivity to large molecules which suggested that altered permeability to large molecules of mutant channels between the supporting cells might be associated to HL (Meşe, Valiunas, Brink, & White, 2008). Moreover, faster intercellular Ca^{2+} signaling was observed in cells coupled by

heteromeric channels containing both Cx26 and Cx30 (Sun *et al*., 2005), suggesting that such channels may mediate and be important to Ca^{2+} signaling in cochlear tissue.

Although HL in Cx30 null mice had been prevented by overexpression of Cx26 (Shoeb Ahmad *et al*., 2007), the distinct localisation of the two connexins in the cochlea (W. Liu, Boström, *et al*., 2009; Y.-P. Liu & Zhao, 2008; Zhao & Yu, 2006) might be a strong indication that the two connexins have non-redundant functions in the normal physiology of the cochlea, further supporting their function beyond the putative role in K^+ recycling.

Therefore, it is very likely that gap junctions play a crucial role in the cochlea by mediating the intercellular diffusion of large biological molecules, as part of biochemical and signaling pathways *in vivo*.

GJB2 mutations

There are over 200 pathogenic *GJB2* mutations described (Stenson *et al*., 2009). Most of them are responsible for recessive NSHL, but a few others are dominant causing either NSHL or HL associated with dermatological disorders (syndromic HL).

The spectrum and the prevalence of *GJB2* mutations vary among different populations. The recessive mutation c.35delG is the most frequent *GJB2* pathogenic mutation identified in NSHL European patients (Antoniadi *et al*., 2000; Gabriel *et al*., 2001; Janecke *et al*., 2002; T. Löppönen *et al*., 2003; Minárik, Feráková, Ficek, Poláková, & Kádasi, 2005; Roux *et al*., 2004; Santos *et al*., 2005; Seeman *et al*., 2004; Šterna *et al*., 2009), in other populations of predominantly European ancestry (Batissoco *et al*., 2009; Dalamón *et al*., 2005; Kelley *et al*., 1998) and in the Mediterranean populations from Algeria (Ammar-Khodja *et al*., 2009), Egypt (Snoeckx, Hassan, Kamal, Van Den Bogaert, & Van Camp, 2005), Lebanon (Mustapha *et al*., 2001), Morocco (Abidi *et al*., 2007) and Tunisia (Masmoudi *et al*., 2000). Two other recessive mutations, c.167delT and c.235delC, are the most frequent *GJB2* pathogenic mutations found in NSHL affected individuals among Ashkenazi Jews (Lerer *et al*., 2000), and in Asian populations from Japan (Abe, Usami, Shinkawa, Kelley, & Kimberling, 2000; Fuse *et al*., 1999), Korea (H. J. Park, Hahn, Chun, Park, & Kim, 2000) and China (Dai *et al*., 2009), respectively.

The c.35delG mutation has a high carrier frequency in Europe in general (1.96%), but is higher (2.86%) in southern Europe (Gasparini *et al*., 2000). This deletion has also a high carrier frequency (2.08%) among Americans of European ancestry (Kelley *et al*., 1998). Regarding the c.167delT mutation, carrier frequencies of 2.78% (Sobe *et al*., 1999), 4.03% (Morell *et al*., 1998) and as high as 7.46% (Lerer *et al*., 2000) have been found among Ashkenazi Jewish individuals. Carrier frequencies for c.235delC mutation range from 0.5% to 2.08% in Eastern Asia (Abe *et al*., 2000; S.-H. Han *et al*., 2008; Hwa *et al*., 2003; Kudo *et al*., 2000; Y. Liu, Ke, Qi, Li, & Zhu, 2002; H. J. Park *et al*., 2000; Shi *et al*., 2004).

One other recessive mutation, p.Arg143Trp, firstly identified in Ghana (Brobby, Müller-Myhsok, & Horstmann, 1998), is the prevalent mutation in congenital, profound NSSHL Ghanese cases (Hamelmann *et al*., 2001). Interestingly, this mutation has a high prevalence among NSHL Japanese cases, compatible with recessive inheritance (Abe *et al*., 2000; Hayashi *et al*., 2011). To a lesser extent, the p.Arg143Trp mutation has been identified in NSHL cases from other Asian countries, such as India (Maheshwari *et al*., 2003; Mani *et al*., 2009), Iran (Hashemzadeh Chaleshtori *et al*., 2005; Najmabadi *et al*., 2005), and Korea (H. J. Park *et al*., 2000). A significant frequency of p.Arg143Trp among moderate to profound NSSHL cases, compatible with recessive inheritance, was also found in Argentina, a country which has received major immigration fluxes from Italy and Spain (Gravina *et al*., 2010). Noteworthy, the p.Arg143Trp was among the most frequent *GJB2* variants detected in North American HL patients of Hispanic ethnicity (Putcha *et al*., 2007).

A different pathogenic mutation, p.Trp24X, was the predominant *GJB2* mutation identified in NSHL cases in India (RamShankar *et al*., 2003), and in Gypsies (an ethnic group that traces back to the Indian subcontinent) from Eastern Slovakia (Minárik *et al*., 2003) and Spain (Álvarez *et al*., 2005). This mutation was also found to have a high carrier frequency (2.4%) in the Indian population (RamShankar *et al*., 2003), and in some subpopulations of Gypsies (Minárik *et al*., 2003; Álvarez *et al*., 2005).

As previously mentioned, due to the high frequency of *GJB2* mutations among NSHL/NSSHL cases in several populations worldwide, this gene is routinely screened for mutations in patients with this pathology. However, only the coding region has been

systematically analysed. Nonetheless, several studies have also searched for mutations in exon 1 (Al-Qahtani *et al*., 2010; Denoyelle *et al*., 1999; Godbole *et al*., 2010; Green *et al*., 1999; Hamelmann *et al*., 2001; Hashemzadeh Chaleshtori *et al*., 2002; Janecke *et al*., 2002; X. Z. Liu, Xia, *et al*., 2002; Mani *et al*., 2009; Marlin *et al*., 2005; Matos *et al*., 2008, 2007, 2011, 2010; Najmabadi *et al*., 2002, 2005; Pollak *et al*., 2008; Prasad, Cucci, Green, & Smith, 2000; RamShankar *et al*., 2003; Riazalhosseini *et al*., 2005; Roux *et al*., 2004; Seeman & Sakmaryová, 2006; Sirmaci, Akcayoz-Duman, & Tekin, 2006; H.-Y. Tang *et al*., 2006; Y. Yuan *et al*., 2009, 2010). The promoter region has also been screened, but in fewer studies (Hashemzadeh Chaleshtori *et al*., 2002; Houseman *et al*., 2001; Matos *et al*., 2008, 2007, 2011, 2010; Pollak *et al*., 2008; Sirmaci *et al*., 2006; Y. Yuan *et al*., 2009, 2010). As a result, a few noncoding pathogenic mutations have been described. Their identification contributed to the elucidation of the genetic etiology of the HL in some patients who harboured only one recessive mutation in the *GJB2* coding region.

The donor splice site c.-23+1G>A (commonly known as IVS1+1G>A) mutation has explained a high proportion of such unelucidated cases in some populations. This mutation, first identified by (Denoyelle *et al*., 1999), and later proven to prevent the transcription of the gene (Shahin *et al*., 2002), has been identified in 45% (9/20) of Czech and 23% (11/47) of Hungarian hearing-impaired individuals with only one *GJB2* coding mutation (Seeman & Sakmaryová, 2006; Tóth *et al*., 2007). In one other study, 50% (8/16) of Turkish HL patients heterozygous for an exon 2 mutation, were also heterozygous for the c.-23+1G>A mutation (Sirmaci *et al*., 2006). Compound heterozygosity between the c.-23+1G>A mutation and a coding *GJB2* mutation has also been detected in patients from other populations (Janecke *et al*., 2002; Medica, Rudolf, Balaban, & Peterlin, 2005; Najmabadi *et al*., 2005; Prasad *et al*., 2000; Y. Yuan *et al*., 2010).

One other noncoding mutation, c.-23G>T, located at the position -1 in respect to the donor splice site, has been recently identified (Mani *et al*., 2009). This mutation, predicted to impair the splice site, was present in *trans* with p.Trp24X, in a severe to profound HL patient from India.

Y. Yuan *et al*. (2010) reported a patient harbouring a novel exon 1 variant, c.-3175C>T, in compound heterozygosity with c.235delC. These variants were most likely in *trans* since the

patient's father was simple heterozygous for the exon 1 variant. The pathogenicity of c.-3175C>T, which was not present in 105 normal-hearing controls, is possible, but still unclear.

A pathogenic mutation within the basal promoter has been found in the context of the present work (Matos *et al*., 2007).

Gross deletions compromising GJB2/GJB6

Pathogenic point mutations in the *GJB2* gene are numerous and are a frequent cause of NSHL. In contrast, *GJB6* point mutations are seemingly a rare cause of this pathology (Bhalla, Sharma, Khandelwal, Panda, & Khullar, 2011; Gürtler, Egenter, Bösch, & Plasilova, 2008; Nahili *et al*., 2008). Nonetheless, a few *GJB6* variants have been identified in some NSHL cases. The *GJB6* mutations p.Thr5Met (Grifa *et al*., 1999), and c.63delG (Pandya, personal communication in Ballana, Ventayol, Rabionet, Gasparini, & Estivill, 2011) have been associated with dominant NSHL, while two other *GJB6* variants, c. 631T>G (p.Cys211Gly) and c.689insA, were identified in compound heterozygosity in two NSSHL cases (Putcha *et al*., 2007). The first one was accompanied by the rare c.110T>C (p.Val37Ala) *GJB2* variant [firstly identified by Azaiez *et al*. (2004), in heterozygosity, in a NSHL patient born to normal-hearing parents; classified as pathogenic (Husami *et al*., 2011)] and the second one by the *GJB2* c.35delG deletion. Y. Yuan *et al*. (2010) found a NSHL patient, with no *GJB2* mutations identified, carrying one other *GJB6* variant, c.404C>A (p.Thr135Lys). While *GJB6* point mutations are rare, two gross deletions, del(*GJB6*-D13S1830) (I. del Castillo *et al*., 2002; Lerer *et al*., 2001; Pallares-Ruiz *et al*., 2002) and del(*GJB6*-D13S1854) (F. J. del Castillo *et al*., 2005), localised upstream of the *GJB2* gene, and disrupting the *GJB6* gene, have been identified and shown to be associated with HL in several populations, most often due to compound heterozygosity with *GJB2* mutations (Angeli, 2008; Cama *et al*., 2009; Chora *et al*., 2010; Cordeiro-Silva *et al*., 2011; Dalamón *et al*., 2005; F. J. del Castillo *et al*., 2005; I. del Castillo *et al*., 2003; Gravina *et al*., 2010; Marlin *et al*., 2005; Pandya *et al*., 2003; Piatto, Bertollo, Sartorato, & Maniglia, 2004; Roux *et al*., 2004; Santos *et al*., 2005; Seeman *et al*., 2005; Taitelbaum-Swead *et al*., 2006). Less frequently, homozygosity for del(*GJB6*-D13S1830), or compound heterozygosity for both deletions, have also been identified in HL patients (Angeli, 2008; F. J. del Castillo *et al*., 2005; I. del Castillo *et al*., 2003; Pandya *et al*.,

2003; Roux *et al*., 2004). Interestingly, (Erbe, Harris, Runge-Samuelson, Flanary, & Wackym, 2004) reported one hearing-impaired child, not harbouring any *GJB2* coding mutations, who was compound heterozygous for del(*GJB6*-D13S1830) and for a *GJB6* coding mutation (p.His124Gln).

Common *et al*. (2005) observed loss of Cx26 expression from the allele bearing the del(*GJB6*-D13S1830) deletion, in ductal sweat gland epithelium, and suggested the existence of a regulatory element of Cx26 expression. Rodriguez-Paris and Schrijver (2009) demonstrated, by qualitative allele-specific RT-PCR analyses using total RNA isolated from buccal ephitelium, that del(*GJB6*-D13S1830) causes loss of expression of *GJB2* gene in *cis*, providing further support for the hypothesis of existence of a *cis*-regulatory element regulating *GJB2*. Later, Rodriguez-Paris, Tamayo, Gelvez, and Schrijver (2011) showed that the del(*GJB6*-D13S1854) *GJB6* deletion, which is smaller than, and included within, the del(*GJB6*-D13S1830) also causes loss of *GJB2* expression in *cis*, as previously suspected.

A third deletion, of 131.4 kbp, del(chr13:19,837,344-19,968,698), localised upstream of *GJB2* and *GJB6* has been identified segregating with HL and in *trans* with c.35delG, in one large American family of German descent. The *GJB2* and *GJB6* expression from alleles bearing the deletion was found to be reduced (Wilch *et al*., 2010, 2006). Wilch *et al*. (2010) hypothesized a similar mechanism for the pathogenic effect of this deletion, and the two deletions truncating *GJB6*, which would involve, in addition to loss or reduction in expression of *GJB6*, the loss of a *GJB2 cis*-regulatory element located within the deleted 95.4 kbp genomic interval shared by the three deletions. The expression of *GJB6* gene might also be regulated by this element, or, instead, a distinct *cis*-regulatory element of this gene might exist and be deleted by del(chr13:19,837,344-19,968,698). Alternatively, the authors suggest that this third deletion could be in linkage disequilibrium (LD) with a pathogenic variant closer to both *GJB2* and *GJB6* that disrupts *cis*-regulatory function of these genes.

Feldmann *et al*. (2009) identified a large deletion, encompassing at least 920 kbp, which completely deletes the three connexin genes *GJA3*, *GJB2* and *GJB6*, as well as at least four other genes. This deletion was identified in *trans* with the c.250G>A (p.Val84Met) *GJB2* mutation in an individual presenting prelingual, profound HL.

Unelucidated GJB2 or GJB6 heterozygotes

In some patients, after searching for mutations in the *GJB2* coding region, splice sites and exon 1, and screening for the del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) *GJB6* deletions, only one recessive *GJB2* coding mutation is identified (F. J. del Castillo *et al*., 2005; I. del Castillo *et al*., 2003). Some of these individuals may indeed just be carriers of a *GJB2* mutant allele but in some other patients a second mutation may exist in other regions of *GJB2* gene, such as the promoter, as shown in this work (Matos *et al*., 2007), the intron or the 3' UTR, or in the *GJB6* gene. The fore mentioned 131.4 kbp or the ~920 kbp *GJB2*/*GJB6* gross deletions, other deletions truncating *GJB2* or *GJB6*, or mutations disrupting a putative upstream regulatory element of these genes, may account for some of these unresolved cases.

GJB2 mutations with unknown or controversial pathogenicity

The pathogenicity of several *GJB2* mutations is not always clear, due to the lack of evidence for or to existing contradictory data regarding the segregation of the mutation with the HL. Examples of such mutations are c.-684_-675del (Houseman *et al*., 2001; Zoll *et al*., 2003), p.Met34Thr (Bicego *et al*., 2006; Cama *et al*., 2009; Feldmann *et al*., 2004; Griffith *et al*., 2000; Houseman *et al*., 2001; Kenna, Wu, Cotanche, Korf, & Rehm, 2001; Pollak *et al*., 2007; Snoeckx, Huygen, *et al*., 2005; B.-L. Wu *et al*., 2002), p.Arg127His (Matos *et al*., 2010), p.Val153Ile (Cryns *et al*., 2004; Dalamón *et al*., 2005; Hashemzadeh Chaleshtori *et al*., 2005; Kenna *et al*., 2001; Marlin *et al*., 2001; RamShankar *et al*., 2003; Snoeckx, Hassan, *et al*., 2005; Snoeckx, Huygen, *et al*., 2005) and p.Gly160Ser (X. Cheng *et al*., 2005; Hashemzadeh Chaleshtori, Farhud, & Patton, 2007; Janecke *et al*., 2002; Löffler *et al*., 2001; Santos *et al*., 2005; Snoeckx, Huygen, *et al*., 2005; H.-Y. Tang *et al*., 2006).

DFNB1 mutations and human evolution

The high frequency of DFNB1-related HL, observed in several populations, has been hypothesized to be due to the combined effects of relaxed selection and assortative mating (Nance & Kearsey, 2004). Furthermore, it has been suggested that a carrier status for Cx26

mutations might confer advantages. Carriers of c.35delG and p.Arg143Trp have been shown to display increased epidermal thickening (Guastalla *et al*., 2009; Meyer *et al*., 2002) which might confer protection against infection. Common, Di, Davies, and Kelsell (2004) observed increased survival of cells expressing the p.Arg143Trp-Cx26 protein, other Cx26 mutants, or the p.Thr5Met-Cx30 mutant, compared with the wild-type proteins, which they suggested could account for the increased epidermal thickening in carriers. Increased cell migration and decreased susceptibility to cellular invasion by the enteric pathogen *Shigella flexneri* have also been shown for cells expressing the p.Arg143Trp-Cx26 mutant protein in comparison to cells expressing wtCx26 (Man *et al*., 2007).

Section 5 - Functional Studies

In order to elucidate the biological effect of Cx26 mutations (being some of them of unclear or controversial pathogenicity), functional studies have been conducted by several investigators. Consequently, a number of mechanisms for pathogenesis associated with mutations in this connexin have been described (reviewed in Hoang Dinh *et al*., 2009).

Mutations in Cx26 have been shown to interfere with protein translation (D'Andrea *et al*., 2002), oligomerization (P. E. M. Martin, Coleman, Casalotti, Forge, & Evans, 1999) and trafficking to the membrane (Bruzzone *et al*., 2003; Choi *et al*., 2009; Choung, Moon, & Park, 2002; Haack *et al*., 2006; Mani *et al*., 2009; P. E. M. Martin *et al*., 1999; Melchionda *et al*., 2005; Oguchi *et al*., 2005), as well as with the intercellular channel conductance (Choi *et al*., 2009; Meşe, Londin, Mui, Brink, & White, 2004; Piazza *et al*., 2005; Y. Zhang *et al*., 2005) and permeability to tracers such as Cascade Blue (Marziano, Casalotti, Portelli, Becker, & Forge, 2003), PI (Choi *et al*., 2009; Y. Zhang *et al*., 2005), LY (Mani *et al*., 2009; Piazza *et al*., 2005) and EtBr (Meşe *et al*., 2008), and to the second messenger IP3 (Beltramello *et al*., 2005; Y. Zhang *et al*., 2005).

There is other group of mutations, associated with skin disease in addition to HL (Keratitis-Ichthyosis-Deafness syndrome), which were observed to cause cell lysis and death through

abnormal hemichannel activity (Gerido, DeRosa, Richard, & White, 2007; J. R. Lee, Derosa, & White, 2009; Montgomery, White, Martin, Turner, & Holland, 2004; Stong, Chang, Ahmad, & Lin, 2006).

The data obtained from the functional studies performed to date allowed the elucidation of the pathogenic mechanism of several Cx26 mutations, as well as a number of mutations in other connexins. Moreover, these studies have shown that, as already mentioned, some Cx26 mutations do not exhibit significant change in ionic permeability, whereas they do display impaired permeability to some large molecules, including the second messenger IP3 (Beltramello *et al*., 2005; Y. Zhang *et al*., 2005). This suggests that these mutations cause HL not by disrupting ionic cycling but possibly by disrupting biochemical and signaling pathways *in vivo*.

It should be noted that care should be taken upon interpreting the results of functional studies since, in some instances, they produce different results. This situation was observed with some *GJB2* mutations, such as p.Val95Met (Beltramello *et al*., 2005; H.-L. Wang *et al*., 2003; Y. Zhang *et al*., 2005), p.Met34Thr (Bicego *et al*., 2006; D'Andrea *et al*., 2002; Palmada *et al*., 2006; Skerrett, Di, Kasperek, Kelsell, & Nicholson, 2004; White, Deans, Kelsell, & Paul, 1998) and p.Arg127His (Thönnissen *et al*., 2002; H.-L. Wang *et al*., 2003).

Section 6 - Aims and outlines of the thesis

The study of genetic deafness in Portuguese families and the molecular diagnosis of patients with sensorineural HL have been conducted by CGBM/BioFIG Deafness Group since 1998.

The work presented on this thesis aimed to two main goals:

a) To continue the study of the *GJB2* gene in Portuguese NSSHL patients, and to extend it in order to also include noncoding regions (promoter, exon 1, donor splice site and 3'UTR);

b) To investigate the functionality of three *GJB2* mutations: two coding mutations for the first time identified during a previous study, and one occurring in the basal promoter, found during this work.

To date, the study of *GJB2* gene in HL cases worldwide has been mostly focused on the coding region. Some studies have also analysed the exon 1 and the splice sites. However, very few studies investigated the promoter and the whole 3'UTR, which are also potential locations for pathogenic mutations.

We have thus analysed the *GJB2* gene in Portuguese HL patients, focusing on:

i) the coding region and acceptor splice site (Chapter 2);

ii) the exon 1 and donor splice site (Chapters 3, 4, 5 and 6);

iii) the basal promoter (Chapters 3, 4, 5 and 6) and the region upstream of the basal promoter (Chapter 6);

iv) the whole 3'UTR (Chapter 6)

The assessment of the functionality of *GJB2* mutations has been essential for the investigation of the biological mechanisms through which these mutations cause HL. Therefore, we studied three *GJB2* mutations, identified in Portuguese HL patients, and which pathogenic effect could not be conclusively demonstrated through the sparse genetic data available (Chapters 3 and 4).

REFERENCES

Aasen, T., & Kelsell, D. P. (2009). Connexins in Skin Biology. In A. L. Harris & D. Locke (Eds.), *Connexins: A Guide* (pp. 307-321). Humana Press. doi:10.1007/978-1-59745-489-6

Abe, S., Usami, S., Shinkawa, H., Kelley, P. M., & Kimberling, W. (2000). Prevalent connexin 26 gene (GJB2) mutations in Japanese. *Journal of Medical Genetics*, *37*(1), 41-43.

Abidi, O., Boulouiz, R., Nahili, H., Ridal, M., Alami, M. N., Tlili, A., Rouba, H., *et al*. (2007). GJB2 (connexin 26) gene mutations in Moroccan patients with autosomal recessive non-syndromic hearing loss and carrier frequency of the common GJB2-35delG mutation. *International Journal of Pediatric Otorhinolaryngology*, *71*(8), 1239-1245.

Ahmad, Shoab, Chen, S., Sun, J., & Lin, X. (2003). Connexins 26 and 30 are co-assembled to form gap junctions in the cochlea of mice. *Biochemical and Biophysical Research Communications*, *307*(2), 362-368.

Ahmad, Shoeb, Tang, W., Chang, Q., Qu, Y., Hibshman, J., Li, Y., Söhl, G., *et al*. (2007). Restoration of connexin26 protein level in the cochlea completely rescues hearing in a mouse model of human connexin30-linked deafness. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(4), 1337-1341.

Al-Qahtani, M. H., Baghlab, I., Chaudhary, A. G., Abuzenadah, A. M., Bamanie, A., Daghistani, K. J., Safieh, M., *et al*. (2010). Spectrum of GJB2 mutations in a cohort of nonsyndromic hearing loss cases from the Kingdom of Saudi Arabia. *Genetic Testing and Molecular Biomarkers*, *14*(1), 79-83.

Ammar-Khodja, F., Faugère, V., Baux, D., Giannesini, C., Léonard, S., Makrelouf, M., Malek, R., *et al*. (2009). Molecular screening of deafness in Algeria: high genetic heterogeneity involving DFNB1 and the Usher loci, DFNB2/USH1B, DFNB12/USH1D and DFNB23/USH1F. *European Journal of Medical Genetics*, *52*(4), 174-179.

Angeli, S. (2008). Phenotype/genotype correlations in a DFNB1 cohort with ethnical diversity. *The Laryngoscope*, *118*(11), 2014-2023.

Antoniadi, T., Gronskov, K., Sand, A., Pampanos, A., Brondum-Nielsen, K., & Petersen, M. B. (2000). Mutation analysis of the GJB2 (connexin 26) gene by DGGE in Greek patients with sensorineural deafness. *Human Mutation*, *16*(1), 7-12.

Azaiez, H., Chamberlin, G. P., Fischer, S. M., Welp, C. L., Prasad, S., Taggart, R. T., del Castillo, I., *et al*. (2004). GJB2: the spectrum of deafness-causing allele variants and their phenotype. *Human Mutation*, *24*(4), 305-311.

Álvarez, A., Del Castillo, I., Villamar, M., Aguirre, L. A., González-Neira, A., López-Nevot, A., Moreno-Pelayo, M. A., *et al*. (2005). High prevalence of the W24X mutation in the gene encoding connexin-26 (GJB2) in Spanish Romani (gypsies) with autosomal recessive non-syndromic hearing loss. *American Journal of Medical Genetics Part A*, *137A*(3), 255-258.

Baker, T. S., Caspar, D. L., Hollingshead, C. J., & Goodenough, D. A. (1983). Gap junction structures. IV. Asymmetric features revealed by low-irradiation microscopy. *The Journal of Cell Biology*, *96*(1), 204-216.

Ballana, E., Ventayol, M., Rabionet, R., Gasparini, P., & Estivill, X. (2011). Deafness mutations in GJB6 (Cx30). *Connexins and deafness Homepage*. Retrieved June 1, 2011, from http://davinci.crg.es/deafness/index.php?seccion=mut_db&db=nonsynd&nonsynd=cx30mut

Batissoco, A. C., Abreu-Silva, R. S., Braga, M. C. C., Lezirovitz, K., Della-Rosa, V., Alfredo, T., Otto, P. A., *et al*. (2009). Prevalence of GJB2 (connexin-26) and GJB6 (connexin-30) mutations in a cohort of 300 Brazilian hearing-impaired individuals: implications for diagnosis and genetic counseling. *Ear and Hearing*, *30*(1), 1-7.

Beltramello, M., Piazza, V., Bukauskas, F. F., Pozzan, T., & Mammano, F. (2005). Impaired permeability to Ins(1,4,5)P3 in a mutant connexin underlies recessive hereditary deafness. *Nature Cell Biology*, *7*(1), 63-69.

Beurg, M., Evans, M. G., Hackney, C. M., & Fettiplace, R. (2006). A large-conductance calcium-selective mechanotransducer channel in mammalian cochlear hair cells. *Journal of Neuroscience*, *26*(43), 10992-11000.

Bhalla, S., Sharma, R., Khandelwal, G., Panda, N. K., & Khullar, M. (2011). Absence of GJB6 mutations in Indian patients with non-syndromic hearing loss. *International Journal of Pediatric Otorhinolaryngology*, *75*(3), 356-359.

Bicego, M., Beltramello, M., Melchionda, S., Carella, M., Piazza, V., Zelante, L., Bukauskas, F. F., *et al*. (2006). Pathogenetic role of the deafness-related M34T mutation of Cx26. *Human Molecular Genetics*, *15*(17), 2569-2587.

Biedron, S., Westhofen, M., & Ilgner, J. (2009). On the number of turns in human cochleae. *Otology & Neurotology*, *30*(3), 414-417.

Brandt, A., Striessnig, J., & Moser, T. (2003). CaV1.3 channels are essential for development and presynaptic activity of cochlear inner hair cells. *Journal of Neuroscience*, *23*(34), 10832-10840.

Brobby, G. W., Müller-Myhsok, B., & Horstmann, R. D. (1998). Connexin 26 R143W mutation associated with recessive nonsyndromic sensorineural deafness in Africa. *The New England Journal of Medicine*, *338*(8), 548-550.

Bruzzone, R., Veronesi, V., Gomès, D., Bicego, M., Duval, N., Marlin, S., Petit, C., *et al*. (2003). Loss-of-function and residual channel activity of connexin26 mutations associated with non-syndromic deafness. *FEBS Letters*, *533*(1-3), 79-88.

Cama, E., Melchionda, S., Palladino, T., Carella, M., Santarelli, R., Genovese, E., Benettazzo, F., *et al*. (2009). Hearing loss features in GJB2 biallelic mutations and GJB2/GJB6 digenic inheritance in a large Italian cohort. *International Journal of Audiology*, *48*(1), 12-17.

Chang, Q., Tang, W., Ahmad, S., Zhou, B., & Lin, X. (2008). Gap Junction Mediated Intercellular Metabolite Transfer in the Cochlea Is Compromised in Connexin30 Null Mice. *PLoS ONE*, *3*(12), e4088.

Cheng, X., Li, L., Brashears, S., Morlet, T., Ng, S. S., Berlin, C., Hood, L., *et al*. (2005). Connexin 26 variants and auditory neuropathy/dys-synchrony among children in schools for the deaf. *American Journal of Medical Genetics Part A*, *139*(1), 13-18.

Choi, S.-Y., Park, H.-J., Lee, K. Y., Dinh, E. H., Chang, Q., Ahmad, S., Lee, S. H., *et al*. (2009). Different functional consequences of two missense mutations in the GJB2 gene associated with non-syndromic hearing loss. *Human Mutation*, *30*(7), E716-E727.

Chora, J. R., Matos, T. D., Martins, J. H., Alves, M. C., Andrade, S. M., Silva, L. F., Ribeiro, C. A., *et al*. (2010). DFNB1-associated deafness in Portuguese cochlear implant users: prevalence and impact on oral outcome. *International Journal of Pediatric Otorhinolaryngology*, *74*(10), 1135-1139.

Choung, Y. H., Moon, S.-K., & Park, H.-J. (2002). Functional study of GJB2 in hereditary hearing loss. *The Laryngoscope*, *112*(9), 1667-1671.

Cohen-Salmon, M., Ott, T., Michel, V., Hardelin, J. P., Perfettini, I., Eybalin, M., Wu, T., *et al*. (2002). Targeted ablation of connexin26 in the inner ear epithelial gap junction network causes hearing impairment and cell death. *Current Biology*, *12*(13), 1106-1111.

Cohen-Salmon, M., Regnault, B., Cayet, N., Caille, D., Demuth, K., Hardelin, J.-P., Janel, N., *et al*. (2007). Connexin30 deficiency causes instrastrial fluid–blood barrier disruption within the cochlear stria vascularis. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(15), 6229-6234.

Common, J., Bitner-Glindzicz, M., O'Toole, E. A., Barnes, M. R., Jenkins, L., Forge, A., & Kelsell, D. P. (2005). Specific loss of connexin 26 expression in ductal sweat gland epithelium associated with the deletion mutation del(GJB6-D13S1830). *Clinical and Experimental Dermatology*, *30*(6), 688-693.

Common, J., Di, W.-L., Davies, D., & Kelsell, D. P. (2004). Further evidence for heterozygote advantage of GJB2 deafness mutations: a link with cell survival. *Journal of Medical Genetics*, *41*(7), 573-575.

Connors, B. W. (2009). Electrical Signaling with Neuronal Gap Junctions. In A. L. Harris & D. Locke (Eds.), *Connexins: A Guide* (pp. 143-164). Humana Press. doi:10.1007/978-1-59745-489-6

Cordeiro-Silva, M. D. F., Barbosa, A., Santiago, M., Provetti, M., Dettogni, R. S., Tovar, T. T., Rabbi-Bortolini, E., *et al*. (2011). Mutation analysis of GJB2 and GJB6 genes in Southeastern Brazilians with hereditary nonsyndromic deafness. *Molecular Biology Reports*, *38*(2), 1309-1313.

Cruciani, V., & Mikalsen, S.-O. (2005). The connexin gene family in mammals. *Biological Chemistry*, *386*(4), 325-332.

Cruciani, V., & Mikalsen, S.-O. (2006). The vertebrate connexin family. *Cellular and Molecular Life Sciences*, *63*(10), 1125-1140.

Cryns, K., Orzan, E., Murgia, A., Huygen, P., Moreno, F., del Castillo, I., Parker, C., *et al*. (2004). A genotype-phenotype correlation for GJB2 (connexin 26) deafness. *Journal of Medical Genetics*, *41*(3), 147-154.

Dai, P., Yu, F., Han, B., Liu, X., Wang, G., Li, Q., Yuan, Y., *et al*. (2009). GJB2 mutation spectrum in 2063 Chinese patients with nonsyndromic hearing impairment. *Journal of Translational Medicine*, *7*(April), 26.

Dalamón, V., Béhèran, A., Diamante, F., Pallares, N., Diamante, V., & Elgoyhen, A. B. (2005). Prevalence of GJB2 mutations and the del (GJB6-D13S1830) in Argentinean non-syndromic deaf patients. *Hearing Research*, *207*, 43-49.

D'Andrea, P., Veronesi, V., Bicego, M., Melchionda, S., Zelante, L., Di Iorio, E., Bruzzone, R., *et al*. (2002). Hearing loss: frequency and functional studies of the most common connexin26 alleles. *Biochemical and Biophysical Research Communications*, *296*(3), 685-691.

del Castillo, F. J., Rodriguez-Ballesteros, M., Alvarez, A., Hutchin, T., Leonardi, E., de Oliveira, C. A., Azaiez, H., *et al*. (2005). A novel deletion involving the connexin-30 gene, del(GJB6-d13s1854), found in trans with mutations in the GJB2 gene (connexin-26) in subjects with DFNB1 non-syndromic hearing impairment. *Journal of Medical Genetics*, *42*(7), 588-594.

del Castillo, I., Moreno-Pelayo, M. A., del Castillo, F. J., Brownstein, Z., Marlin, S., Adina, Q., Cockburn, D. J., *et al*. (2003). Prevalence and evolutionary origins of the del(GJB6-D13S1830) mutation in the DFNB1 locus in hearing-impaired subjects: a multicenter study. *The American Journal of Human Genetics*, *73*(6), 1452-1458.

del Castillo, I., Villamar, M., Moreno-Pelayo, M. A., del Castillo, F. J., Alvarez, A., Tellería, D., Menéndez, I., *et al*. (2002). A deletion involving the connexin 30 gene in nonsyndromic hearing impairment. *The New England Journal of Medicine*, *346*(4), 243-249.

Denoyelle, F., Marlin, S., Weil, D., Moatti, L., Chauvin, P., Garabédian, E. N., & Petit, C. (1999). Clinical features of the prevalent form of childhood deafness, DFNB1, due to a connexin-26 gene defect: implications for genetic counselling. *The Lancet*, *353*(9161), 1298-1303.

Diez, J. A., Ahmad, S., & Evans, W. H. (1999). Assembly of heteromeric connexons in guinea-pig liver en route to the Golgi apparatus, plasma membrane and gap junctions. *The Federation of European Biochemical Societies Journal*, *262*(1), 142-148.

Encyclopædia Britannica Inc. (n.d.). ear: structure of the human ear. Retrieved November 17, 2011, from http://www.britannica.com/bps/media-view/530/0/0/0

Erbe, C. B., Harris, K. C., Runge-Samuelson, C. L., Flanary, V. A., & Wackym, P. A. (2004). Connexin 26 and connexin 30 mutations in children with nonsyndromic hearing loss. *The Laryngoscope*, *114*(4), 607-611.

Essenfelder, G. M., Larderet, G., Waksman, G., & Lamartine, J. (2005). Gene structure and promoter analysis of the human GJB6 gene encoding connexin 30. *Gene*, *350*(1), 33-40.

Feldmann, D., Denoyelle, F., Loundon, N., Weil, D., Garabedian, E.-N., Couderc, R., Joannard, A., *et al*. (2004). Clinical evidence of the nonpathogenic nature of the M34T variant in the connexin 26 gene. *European Journal of Human Genetics*, *12*(4), 279-284.

Feldmann, D., Le Maréchal, C., Jonard, L., Thierry, P., Czajka, C., Couderc, R., Ferec, C., *et al*. (2009). A new large deletion in the DFNB1 locus causes nonsyndromic hearing loss. *European Journal of Medical Genetics*, *52*(4), 195-200.

Forge, A., Becker, D., Casalotti, S., Edwards, J., Marziano, N., & Nevill, G. (2003). Gap junctions in the inner ear: comparison of distribution patterns in different vertebrates and assessement of connexin composition in mammals. *Journal of Comparative Neurology*, *467*(2), 207-231.

Fuchs, P. A., Glowatzki, E., & Moser, T. (2003). The afferent synapse of cochlear hair cells. *Current Opinion in Neurobiology*, *13*(4), 452-458.

Fuse, Y., Doi, K., Hasegawa, T., Sugii, A., Hibino, H., & Kubo, T. (1999). Three novel connexin26 gene mutations in autosomal recessive non-syndromic deafness. *NeuroReport*, *10*(9), 1853-1857.

Gabriel, H., Kupsch, P., Sudendey, J., Winterhager, E., Jahnke, K., & Lautermann, J. (2001). Mutations in the connexin26/GJB2 gene are the most common event in non-syndromic hearing loss among the German population. *Human Mutation*, *17*(6), 521-522.

Gasparini, P., Rabionet, R., Barbujani, G., Melchionda, S., Petersen, M., Brøndum-Nielsen, K., Metspalu, A., *et al*. (2000). High carrier frequency of the 35delG deafness mutation in European populations. *European Journal of Human Genetics*, *8*(1), 19-23.

Gerido, D. A., DeRosa, A. M., Richard, G., & White, T. W. (2007). Aberrant hemichannel properties of Cx26 mutations causing skin disease and deafness. *American Journal of Physiology Cell Physiology*, *293*(1), C337-C345.

Glowatzki, E., Cheng, N., Hiel, H., Yi, E., Tanaka, K., Ellis-Davies, G. C. R., Rothstein, J. D., *et al*. (2006). The glutamate-aspartate transporter GLAST mediates glutamate uptake at inner hair cell afferent synapses in the mammalian cochlea. *Journal of Neuroscience*, *26*(29), 7659-7664.

Godbole, K., Hemavathi, J., Vaid, N., Pandit, A. N., Sandeep, M. N., & Chandak, G. R. (2010). Low prevalence of GJB2 mutations in non-syndromic hearing loss in Western India. Indian *Journal of Otolaryngology and Head & Neck Surgery*, *62*(1), 60-63.

Gravina, L. P., Foncuberta, M. E., Prieto, M. E., Garrido, J., Barreiro, C., & Chertkoff, L. (2010). Prevalence of DFNB1 mutations in Argentinean children with non-syndromic deafness. Report of a novel mutation in GJB2. *International Journal of Pediatric Otorhinolaryngology*, *74*(3), 250-254.

Green, G. E., Scott, D. A., McDonald, J. M., Woodworth, G. G., Sheffield, V. C., & Smith, R. (1999). Carrier rates in the midwestern United States for GJB2 mutations causing inherited deafness. *Jama The Journal Of The American Medical Association*, *281*(23), 2211-2216.

Grifa, A., Wagner, C. A., D'Ambrosio, L., Melchionda, S., Bernardi, F., Lopez-Bigas, N., Rabionet, R., *et al*. (1999). Mutations in GJB6 cause nonsyndromic autosomal dominant deafness at DFNA3 locus. *Nature Genetics*, *23*(1), 16-18.

Griffith, A. J., Chowdhry, A. A., Kurima, K., Hood, L. J., Keats, B., Berlin, C. I., Morell, R., *et al*. (2000). Autosomal recessive nonsyndromic neurosensory deafness at DFNB1 not associated with the compound-heterozygous GJB2 (connexin 26) genotype M34T/167delT. *The American Journal of Human Genetics*, *67*(3), 745-749.

Guastalla, P., Guerci, V. I., Fabretto, A., Faletra, F., Grasso, D. L., Zocconi, E., Stefanidou, D., *et al*. (2009). Detection of epidermal thickening in GJB2 carriers with epidermal US. *Radiology*, *251*(1), 280-286.

Gürtler, N., Egenter, C., Bösch, N., & Plasilova, M. (2008). Mutation analysis of the Cx26, Cx30, and Cx31 genes in autosomal recessive nonsyndromic hearing impairment. *Acta Otolaryngologica*, *128*(10), 1056-1062.

Haack, B., Schmalisch, K., Palmada, M., Böhmer, C., Kohlschmidt, N., Keilmann, A., Zechner, U., *et al*. (2006). Deficient membrane integration of the novel p.N14D-GJB2 mutant associated with non-syndromic hearing impairment. *Human Mutation*, *27*(11), 1158-1159.

Haefliger, J. A., Bruzzone, R., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., & Paul, D. L. (1992). Four novel members of the connexin family of gap junction proteins. Molecular cloning, expression, and chromosome mapping. *The Journal of Biological Chemistry*, *267*(3), 2057-2064.

Hamelmann, C., Amedofu, G. K., Albrecht, K., Muntau, B., Brobby, G. W., & Horstmann, R. D. (2001). Pattern of Connexin 26 (GJB2) Mutations Causing Sensorineural Hearing Impairment in Ghana. *Blood*, *428*(March), 1-6.

Han, S.-H., Park, H.-J., Kang, E.-J., Ryu, J.-S., Lee, A., Yang, Y.-H., & Lee, K.-R. (2008). Carrier frequency of GJB2 (connexin-26) mutations causing inherited deafness in the Korean population. *Journal of Human Genetics*, *53*(11-12), 1022-1028.

Harris, A. L. (2001). Emerging issues of connexin channels: biophysics fills the gap. *Quarterly Reviews of Biophysics*, *34*(3), 325-472.

Harris, A. L. (2007). Connexin channel permeability to cytoplasmic molecules. *Progress in Biophysics and Molecular Biology*, *94*(1-2), 120-143.

Harris, A. L., & Locke, D. (Eds.). (2009). *Connexins: A Guide* (p. 576). New York, NY 10013, USA: Humana Press. doi:10.1007/978-1-59745-489-6

Hashemzadeh Chaleshtori, M., Farhud, D. D., & Patton, M. A. (2007). Familial and Sporadic GJB2-Related Deafness in Iran: Review of Gene Mutations. *Iranian Journal of Public Health*, *36*(1), 1-14.

Hashemzadeh Chaleshtori, M., Farhud, D. D., Taylor, R., Hadavi, V., Patton, M. A., & Afzal, A. R. (2002). Deafness –Associated Connexin 26 Gene (GJB2) Mutations in Iranian Population. *Iranian Journal of Public Health*, *31*(3-4), 75-79.

Hashemzadeh Chaleshtori, M., Rad, L. H., Dolati, M., Sasanfar, R., Hoseinipour, A., Zohour, M. M., Pourjafari, H., *et al*. (2005). Frequencies of Mutations in the Connexin 26 Gene (GJB2) in Two Populations of Iran (Tehran and Tabriz). *Iranian Journal of Public Health*, *34*(1), 1-7.

Hayashi, C., Funayama, M., Li, Y., Kamiya, K., Kawano, A., Suzuki, M., Hattori, N., *et al*. (2011). Prevalence of GJB2 causing recessive profound non-syndromic deafness in Japanese children. *International Journal of Pediatric Otorhinolaryngology*, *75*(2), 211-214.

He, D. S., Jiang, J. X., Taffet, S. M., & Burt, J. M. (1999). Formation of heteromeric gap junction channels by connexins 40 and 43 in vascular smooth muscle cells. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(11), 6495-6500.

Hearing Central LLC. (n.d.). How the Ear Works - With Images and Graphics. Retrieved December 15, 2011, from http://www.hearingcentral.com/howtheearworks.asp

Hibino, H., & Kurachi, Y. (2006). Molecular and physiological bases of the K+ circulation in the mammalian inner ear. *Physiology*, *21*(5), 336-45.

Hoang Dinh, E., Ahmad, S., Chang, Q., Tang, W., Stong, B., & Lin, X. (2009). Diverse deafness mechanisms of connexin mutations revealed by studies using in vitro approaches and mouse models. *Brain Research*, *1277*, 52-69.

Houseman, M., Ellis, L., Pagnamenta, A., Di, W., Rickard, S., Osborn, A., Dahl, H., *et al*. (2001). Genetic analysis of the connexin-26 M34T variant: identification of genotype M34T/M34T segregating with mild-moderate non-syndromic sensorineural hearing loss. *Journal of Medical Genetics*, *38*(1), 20-25.

Husami *et al*. (2011). No Title. ***Public** CCHMC Molecular Genetics Laboratory Mutation Database - gap junction protein, beta 2, 26kDa (GJB2)*. Retrieved November 4, 2011, from https://research.cchmc.org/LOVD/variants.php?select_db=GJB2&action=search_all&search_Variant/DNA=c.110T>C

Hwa, H.-L., Ko, T.-M., Hsu, C.-J., Huang, C.-H., Chiang, Y.-L., Oong, J.-L., Chen, C.-C., *et al*. (2003). Mutation spectrum of the connexin 26 (GJB2) gene in Taiwanese patients with prelingual deafness. *Genetics in Medicine*, *5*(3), 161-165.

Jagger, D. J., & Forge, A. (2006). Compartmentalized and signal-selective gap junctional coupling in the hearing cochlea. *Journal of Neuroscience*, *26*(4), 1260-1268.

Janecke, A. R., Hirst-Stadlmann, A., Günther, B., Utermann, B., Müller, T., Löffler, J., Utermann, G., *et al*. (2002). Progressive hearing loss, and recurrent sudden sensorineural hearing loss associated with GJB2 mutations--phenotypic spectrum and frequencies of GJB2 mutations in Austria. *Human Genetics*, *111*(2), 145-153.

Jiang, J. X., & Goodenough, D. A. (1996). Heteromeric connexons in lens gap junction channels. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(3), 1287-1291.

Kanno, S., & Saffitz, J. E. (2001). The role of myocardial gap junctions in electrical conduction and arrhythmogenesis. *Cardiovascular Pathology*, *10*(4), 169-177.

Kelley, P. M., Harris, D. J., Comer, B. C., Askew, J. W., Fowler, T., Smith, S. D., & Kimberling, W. J. (1998). Novel mutations in the connexin 26 gene (GJB2) that cause autosomal recessive (DFNB1) hearing loss. *The American Journal of Human Genetics*, *62*(4), 792-799.

Kenna, M., Wu, B. L., Cotanche, D. A., Korf, B. R., & Rehm, H. L. (2001). Connexin 26 studies in patients with sensorineural hearing loss. *Archives of Otolaryngology Head & Neck Surgery*, *127*(9), 1037-1042.

Kenneson, A., Van Naarden Braun, K., & Boyle, C. (2002). GJB2 (connexin 26) variants and nonsyndromic sensorineural hearing loss: a HuGE review. *Genetics in Medicine*, *4*(4), 258-274.

Kikuchi, T., Adams, J. C., Miyabe, Y., So, E., & Kobayashi, T. (2000). Potassium ion recycling pathway via gap junction systems in the mammalian cochlea and its interruption in hereditary nonsyndromic deafness. *Medical Electron Microscopy*, *33*(2), 51-56.

Kikuchi, T., Kimura, R. S., Paul, D. L., & Adams, J. C. (1995). Gap junctions in the rat cochlea: immunohistochemical and ultrastructural analysis. *Anatomy and Embryology*, *191*(2), 101-118.

Kikuchi, T., Kimura, R. S., Paul, D. L., Takasaka, T., & Adams, J. C. (2000). Gap junction systems in the mammalian cochlea. *Brain Research Brain Research Reviews*, *32*(1), 163-166.

Kudo, T., Ikeda, K., Kure, S., Matsubara, Y., Oshima, T., Watanabe, K. I., Kawase, T., *et al*. (2000). Novel mutations in the connexin 26 gene (GJB2) responsible for childhood deafness in the Japanese population. *American Journal of Medical Genetics*, *90*(2), 141-145.

Kumar, N. M., & Gilula, N. B. (1996). The gap junction communication channel. *Cell*, *84*(3), 381-388.

Langlois, S., Maher, A. C., Manias, J. L., Shao, Q., Kidder, G. M., & Laird, D. W. (2007). Connexin levels regulate keratinocyte differentiation in the epidermis. *The Journal of Biological Chemistry*, *282*(41), 30171-30180.

Lautermann, J., ten Cate, W. J., Altenhoff, P., Grümmer, R., Traub, O., Frank, H., Jahnke, K., *et al*. (1998). Expression of the gap-junction connexins 26 and 30 in the rat cochlea. *Cell and Tissue Research*, *294*(3), 415-420.

LeMasurier, M., & Gillespie, P. G. (2005). Hair-cell mechanotransduction and cochlear amplification. *Neuron*, *48*(3), 403-415.

Lee, J. R., Derosa, A. M., & White, T. W. (2009). Connexin mutations causing skin disease and deafness increase hemichannel activity and cell death when expressed in Xenopus oocytes. *The Journal of Investigative Dermatology*, *129*(4), 870-878.

Lerer, I., Sagi, M., Ben-Neriah, Z., Wang, T., Levi, H., & Abeliovich, D. (2001). A deletion mutation in GJB6 cooperating with a GJB2 mutation in trans in non-syndromic deafness: A novel founder mutation in Ashkenazi Jews. *Human Mutation*, *18*(5), 460.

Lerer, I., Sagi, M., Malamud, E., Levi, H., Raas-Rothschild, A., & Abeliovich, D. (2000). Contribution of connexin 26 mutations to nonsyndromic deafness in Ashkenazi patients and the variable phenotypic effect of the mutation 167delT. *American Journal of Medical Genetics*, *95*(1), 53-56.

Liu, W., Boström, M., Kinnefors, A., & Rask-Andersen, H. (2009). Unique expression of connexins in the human cochlea. *Hearing Research*, *250*(1-2), 55-62.

Liu, X. Z., Xia, X. J., Ke, X. M., Ouyang, X. M., Du, L. L., Liu, Y. H., Angeli, S., *et al*. (2002). The prevalence of connexin 26 (GJB2) mutations in the Chinese population. *Human Genetics*, *111*(4-5), 394-397.

Liu, X. Z., Yuan, Y., Yan, D., Ding, E. H., Ouyang, X. M., Fei, Y., Tang, W., *et al*. (2009). Digenic inheritance of non-syndromic deafness caused by mutations at the gap junction proteins Cx26 and Cx31. *Human Genetics*, *125*(1), 53-62.

Liu, Y., Ke, X., Qi, Y., Li, W., & Zhu, P. (2002). Connexin26 gene (GJB2): prevalence of mutations in the Chinese population. *Journal of Human Genetics*, *47*(12), 688-690.

Liu, Y.-P., & Zhao, H.-B. (2008). Cellular characterization of Connexin26 and Connnexin30 expression in the cochlear lateral wall. *Cell and Tissue Research*, *333*(3), 395-403.

Locke, D., Jamieson, S., Stein, T., Liu, J., Hodgkins, M. B., Harris, A. L., & Gusterson, B. (2007). Nature of Cx30-containing channels in the adult mouse mammary gland. *Cell and Tissue Research*, *328*(1), 97-107.

Locke, D., Perusinghe, N., Newman, T., Jayatilake, H., Evans, W. H., & Monaghan, P. (2000). Developmental expression and assembly of connexins into homomeric and heteromeric gap junction hemichannels in the mouse mammary gland. *Journal of Cellular Physiology*, *183*(2), 228-237.

Löffler, J., Nekahm, D., Hirst-Stadlmann, A., Günther, B., Menzel, H. J., Utermann, G., & Janecke, A. R. (2001). Sensorineural hearing loss and the incidence of Cx26 mutations in Austria. *European Journal of Human Genetics*, *9*(3), 226-230.

Löppönen, T., Väisänen, M.-L., Luotonen, M., Allinen, M., Uusimaa, J., Lindholm, P., Mäki-Torkko, E., *et al*. (2003). Connexin 26 mutations and nonsyndromic hearing impairment in northern Finland. *The Laryngoscope*, *113*(10), 1758-1763.

Maheshwari, M., Vijaya, R., Ghosh, M., Shastri, S., Kabra, M., & Menon, P. S. N. (2003). Screening of families with autosomal recessive non-syndromic hearing impairment (ARNSHI) for mutations in GJB2 gene: Indian scenario. *American Journal of Medical Genetics Part A*, *120A*(2), 180-184.

Makowski, L., Caspar, D. L. D., Phillips, W. C., & Goodenough, D. A. (1977). Gap junction structures. II. Analysis of the x-ray diffraction data. *The Journal of Cell Biology*, *74*(2), 629-645.

Man, Y. K. S., Trolove, C., Tattersall, D., Thomas, A. C., Papakonstantinopoulou, A., Patel, D., Scott, C., *et al*. (2007). A deafness-associated mutant human connexin 26 improves the epithelial barrier In vitro. *The Journal of Membrane Biology*, *218*(1-3), 29-37.

Mani, R. S., Ganapathy, A., Jalvi, R., Srikumari Srisailapathy, C. R., Malhotra, V., Chadha, S., Agarwal, A., *et al*. (2009). Functional consequences of novel connexin 26 mutations associated with hereditary hearing loss. *European Journal of Human Genetics*, *17*(4), 502-509.

Marcus, D. C., Wu, T., Wangemann, P., & Kofuji, P. (2002). KCNJ10 (Kir4.1) potassium channel knockout abolishes endocochlear potential. *American Journal of Physiology Cell Physiology*, *282*(2), C403-C407.

Marlin, S., Feldmann, D., Blons, H., Loundon, N., Rouillon, I., Albert, S., Chauvin, P., *et al*. (2005). GJB2 and GJB6 mutations: genotypic and phenotypic correlations in a large cohort of hearing-impaired patients. *Archives of Otolaryngology Head & Neck Surgery*, *131*(6), 481-487.

Marlin, S., Garabédian, E. N., Roger, G., Moatti, L., Matha, N., Lewin, P., Petit, C., *et al*. (2001). Connexin 26 gene mutations in congenitally deaf children: pitfalls for genetic counseling. *Archives of Otolaryngology Head & Neck Surgery*, *127*(8), 927-933.

Martin, P. E. M., Coleman, S. L., Casalotti, S., Forge, A., & Evans, W. H. (1999). Properties of connexin26 gap junctional proteins derived from mutations associated with non-syndromal heriditary deafness. *Human Molecular Genetics*, *8*(13), 2369-2376.

Marziano, N., Casalotti, S. O., Portelli, A. E., Becker, D. L., & Forge, A. (2003). Mutations in the gene for connexin 26 (GJB2) that cause hearing loss have a dominant negative effect on connexin 30. *Human Molecular Genetics*, *12*(8), 805-812.

Masmoudi, S., Elgaied-Boulila, A., Kassab, I., Arab, S. B., Blanchard, S., Bouzoita, J., Drira, M., *et al*. (2000). Determination of the frequency of connexin26 mutations in inherited sensorineural deafness and carrier rates in the Tunisian population using DGGE. *Journal of Medical Genetics*, *37*(11), e39.

Mathias, R. T., White, T. W., & Gong, X. (2010). Lens gap junctions in growth, differentiation, and homeostasis. *Physiological Reviews*, *90*(1), 179-206.

Matos, T. D., Caria, H., Simões-Teixeira, H., Aasen, T., Dias, O., Andrea, M., Kelsell, D. P., *et al*. (2008). A novel M163L mutation in connexin 26 causing cell death and associated with autosomal dominant hearing loss. *Hearing Research*, *240*(1-2), 87-92.

Matos, T. D., Caria, H., Simões-Teixeira, H., Aasen, T., Nickel, R., Jagger, D. J., O'Neill, A., *et al*. (2007). A novel hearing-loss-related mutation occurring in the GJB2 basal promoter. *Journal of Medical Genetics*, *44*(11), 721-725.

Matos, T. D., Simões-Teixeira, H., Caria, H., Cascão, R., Rosa, H., O'Neill, A., Dias, Ó., *et al*. (2011). Assessing Noncoding Sequence Variants of GJB2 for Hearing Loss Association. *Genetics Research International*, *2011*. doi:10.4061/2011/827469

Matos, T. D., Simões-Teixeira, H., Caria, H., Rosa, H., O'Neill, A., & Fialho, G. (2010). The controversial p.Arg127His mutation in GJB2: report on three Portuguese hearing loss family cases. *Genetic Testing and Molecular Biomarkers*, *14*(1), 141-144.

Medica, I., Rudolf, G., Balaban, M., & Peterlin, B. (2005). C.35delG/ GJB2 and del(GJB6-D13S1830) mutations in Croatians with prelingual non-syndromic hearing impairment. *BMC Ear Nose and Throat Disorders*, *5*, 11.

Melchionda, S., Bicego, M., Marciano, E., Franze, A., Morgutti, M., Bortone, G., Zelante, L., *et al*. (2005). Functional characterization of a novel Cx26 (T55N) mutation associated to non-syndromic hearing loss. *Biochemical and Biophysical Research Communications*, *337*(3), 799-805.

Meyer, C. G., Amedofu, G. K., Brandner, J. M., Pohland, D., Timmann, C., & Horstmann, R. D. (2002). Selection for deafness? *Nature Medicine*, *8*(12), 1332-1333.

Meşe, G., Londin, E., Mui, R., Brink, P. R., & White, T. W. (2004). Altered gating properties of functional Cx26 mutants associated with recessive non-syndromic hearing loss. *Human Genetics*, *115*(3), 191-199.

Meşe, G., Valiunas, V., Brink, P. R., & White, T. W. (2008). Connexin26 deafness associated mutations show altered permeability to large cationic molecules. *American Journal of Physiology Cell Physiology*, *295*(4), C966-C974.

Minárik, G., Ferák, V., Feráková, E., Ficek, A., Poláková, H., & Kádasi, L. (2003). High frequency of GJB2 mutation W24X among Slovak Romany (Gypsy) patients with non-syndromic hearing loss (NSHL). *General Physiology and Biophysics*, *22*(4), 549-556.

Minárik, G., Feráková, E., Ficek, A., Poláková, H., & Kádasi, L. (2005). GJB2 gene mutations in Slovak hearing-impaired patients of Caucasian origin: spectrum, frequencies and SNP analysis. *Clinical Genetics*, *68*(6), 554-557.

Montgomery, J. R., White, T. W., Martin, B. L., Turner, M. L., & Holland, S. M. (2004). A novel connexin 26 gene mutation associated with features of the keratitis-ichthyosis-deafness syndrome and the follicular occlusion triad. *Journal of the American Academy of Dermatology*, *51*(3), 377-382.

Morell, R., Kim, H. J., Hood, L. J., Goforth, L., Friderici, K., Fisher, R., Van Camp, G., *et al*. (1998). Mutations in the connexin 26 gene (GJB2) among Ashkenazi Jews with nonsyndromic recessive deafness. *The New England Journal of Medicine*, *339*(21), 1500-1505.

Moser, T., & Beutner, D. (2000). Kinetics of exocytosis and endocytosis at the cochlear inner hair cell afferent synapse of the mouse. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(2), 883-888.

Mustapha, M., Salem, N., Delague, V., Chouery, E., Ghassibeh, M., Rai, M., Loiselet, J., *et al*. (2001). Autosomal recessive non-syndromic hearing loss in the Lebanese population: prevalence of the 30delG mutation

and report of two novel mutations in the connexin 26 (GJB2) gene. *Journal of Medical Genetics*, *38*(10), e36.

Møller, A. R. (2006). *Hearing: anatomy, physiology, and disorders of the auditory system* (2nd ed., p. 328). Oxford: Academic Press.

Nahili, H., Ridal, M., Boulouiz, R., Abidi, O., Imken, L., Rouba, H., Alami, M. N., *et al*. (2008). Absence of GJB3 and GJB6 mutations in Moroccan familial and sporadic patients with autosomal recessive non-syndromic deafness. *International Journal of Pediatric Otorhinolaryngology*, *72*(11), 1633-1636.

Najmabadi, H., Cucci, R. A., Sahebjam, S., Kouchakian, N., Farhadi, M., Kahrizi, K., Arzhangi, S., *et al*. (2002). GJB2 mutations in Iranians with autosomal recessive non-syndromic sensorineural hearing loss. *Human Mutation*, *19*(5), 572.

Najmabadi, H., Nishimura, C., Kahrizi, K., Riazalhosseini, Y., Malekpour, M., Daneshi, A., Farhadi, M., *et al*. (2005). GJB2 mutations: passage through Iran. *American Journal of Medical Genetics Part A*, *133A*(2), 132-137.

Nance, W. E., & Kearsey, M. J. (2004). Relevance of connexin deafness (DFNB1) to human evolution. *The American Journal of Human Genetics*, *74*(6), 1081-1087.

Nin, F., Hibino, H., Doi, K., Suzuki, T., Hisa, Y., & Kurachi, Y. (2008). The endocochlear potential depends on two K+ diffusion potentials and an electrical barrier in the stria vascularis of the inner ear. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(5), 1751-1756.

Oguchi, T., Ohtsuka, A., Hashimoto, S., Oshima, A., Abe, S., Kobayashi, Y., Nagai, K., *et al*. (2005). Clinical features of patients with GJB2 (connexin 26) mutations: severity of hearing loss is correlated with genotypes and protein expression patterns. *Journal of Human Genetics*, *50*(2), 76-83.

Ortolano, S., Di Pasquale, G., Crispino, G., Anselmi, F., Mammano, F., & Chiorini, J. A. (2008). Coordinated control of connexin 26 and connexin 30 at the regulatory and functional level in the inner ear. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(48), 18776-18781.

Pallares-Ruiz, N., Blanchet, P., Mondain, M., Claustres, M., & Roux, A.-F. (2002). A large deletion including most of GJB6 in recessive non syndromic deafness: a digenic effect? *European Journal of Human Genetics*, *10*(1), 72-76.

Palmada, M., Schmalisch, K., Böhmer, C., Schug, N., Pfister, M., Lang, F., & Blin, N. (2006). Loss of function mutations of the GJB2 gene detected in patients with DFNB1-associated hearing impairment. *Neurobiology of Disease*, *22*(1), 112-118.

Panchin, Y. (2005). Evolution of gap junction proteins--the pannexin alternative. *Journal of Experimental Biology*, *208*(Pt 8), 1415-1419.

Pandya, A., Arnos, K. S., Xia, X. J., Welch, K. O., Blanton, S. H., Friedman, T. B., Garcia Sanchez, G., *et al*. (2003). Frequency and distribution of GJB2 (connexin 26) and GJB6 (connexin 30) mutations in a large North American repository of deaf probands. *Genetics in Medicine*, *5*(4), 295-303.

Park, H. J., Hahn, S. H., Chun, Y. M., Park, K., & Kim, H. N. (2000). Connexin26 mutations associated with nonsyndromic hearing loss. *The Laryngoscope*, *110*(9), 1535-1538.

Perkins, G., Goodenough, D., & Sosinsky, G. (1997). Three-dimensional structure of the gap junction connexon. *Biophysical Journal*, *72*(2 Pt 1), 533-544.

Perkins, G., Goodenough, D., & Sosinsky, G. (1998). Formation of the gap junction intercellular channel requires a 30 degree rotation for interdigitating two apposing connexons. *Journal of Molecular Biology*, *277*(2), 171-177.

Petit, C., *et al*. (n.d.). The Project. *EuroHear*. Retrieved May 11, 2011, from http://www.eurohear.org/index.php?option=com_content&task=blogcategory&id=3&Itemid=65http://www.eurohear.org/index.php?option=com_content&task=blogcategory&id=3&Itemid=65

Piatto, V. B., Bertollo, E. M. G., Sartorato, E. L., & Maniglia, J. V. (2004). Prevalence of the GJB2 mutations and the del(GJB6-D13S1830) mutation in Brazilian patients with deafness. *Hearing Research*, *196*(1-2), 87-93.

Piazza, V., Beltramello, M., Menniti, M., Colao, E., Malatesta, P., Argento, R., Chiarella, G., *et al*. (2005). Functional analysis of R75Q mutation in the gene coding for Connexin 26 identified in a family with nonsyndromic hearing loss. *Clinical Genetics*, *68*(2), 161-166.

Pollak, A., Mueller-Malesinska, M., Skórka, A., Kostrzewa, G., Ołdak, M., Korniszewski, L., Skarzýński, H., *et al*. (2008). GJB2 and hearing impairment: promoter defects do not explain the excess of monoallelic mutations. *Journal of Medical Genetics*, *45*(9), 607-608.

Pollak, A., Skórka, A., Mueller-Malesińska, M., Kostrzewa, G., Kisiel, B., Waligóra, J., Krajewski, P., *et al*. (2007). M34T and V37I mutations in GJB2 associated hearing impairment: evidence for pathogenicity and reduced penetrance. *American Journal of Medical Genetics Part A*, *143A*(21), 2534-2543.

Prasad, S., Cucci, R. A., Green, G. E., & Smith, R. (2000). Genetic testing for hereditary hearing loss: connexin 26 (GJB2) allele variants and two novel deafness-causing mutations (R32C and 645-648delTAGA). *Human Mutation*, *16*(6), 502-508.

Putcha, G. V., Bejjani, B. A., Bleoo, S., Booker, J. K., Carey, J. C., Carson, N., Das, S., *et al*. (2007). A multicenter study of the frequency and distribution of GJB2 and GJB6 mutations in a large North American cohort. *Genetics in Medicine*, *9*(7), 413-426.

RamShankar, M., Girirajan, S., Dagan, O., Ravi, S., Jalvi, R., Rangasayee, R., Avraham, K., *et al*. (2003). Contribution of connexin26 (GJB2) mutations and founder effect to non-syndromic hearing loss in India. *Journal of Medical Genetics*, *40*(5), e68.

Riazalhosseini, Y., Nishimura, C., Kahrizi, K., Shafeghati, Y., Daneshi, A., Jogataie, M.-T., Mohseni, M., *et al*. (2005). Delta (GJB6-D13S1830) is not a common cause of nonsyndromic hearing loss in the Iranian population. *Archives of Iranian Medicine*, *8*(2), 104-108.

Ricci, A. J., Kennedy, H. J., Crawford, A. C., & Fettiplace, R. (2005). The transduction channel filter in auditory hair cells. *Journal of Neuroscience*, *25*(34), 7831-7839.

Robles, L., & Ruggero, M. A. (2001). Mechanics of the mammalian cochlea. *Physiological Reviews*, *81*(3), 1305-1352.

Rodriguez-Paris, J., & Schrijver, I. (2009). The digenic hypothesis unraveled: the GJB6 del(GJB6-D13S1830) mutation causes allele-specific loss of GJB2 expression in cis. *Biochemical and Biophysical Research Communications*, *389*(2), 354-359.

Rodriguez-Paris, J., Tamayo, M. L., Gelvez, N., & Schrijver, I. (2011). Allele-Specific Impairment of GJB2 Expression by GJB6 Deletion del(GJB6-D13S1854). *PLoS ONE*, *6*(6), 7.

Roux, A.-F., Pallares-Ruiz, N., Vielle, A., Faugère, V., Templin, C., Leprevost, D., Artières, F., *et al*. (2004). Molecular epidemiology of DFNB1 deafness in France. *BMC Medical Genetics*, *5*, 5.

Santos, R. L. P., Aulchenko, Y. S., Huygen, P. L. M., Van Der Donk, K. P., De Wijs, I. J., Kemperman, M. H., Admiraal, R. J. C., *et al*. (2005). Hearing impairment in Dutch patients with connexin 26 (GJB2) and connexin 30 (GJB6) mutations. *International Journal of Pediatric Otorhinolaryngology*, *69*(2), 165-174.

Sasakura, Y., Shoguchi, E., Takatori, N., Wada, S., Meinertzhagen, I. A., Satou, Y., & Satoh, N. (2003). A genomewide survey of developmentally relevant genes in Ciona intestinalis. X. Genes for cell junctions and extracellular matrix. *Development Genes and Evolution*, *213*(5-6), 303-313.

Schrijver, I. (2004). Hereditary non-syndromic sensorineural hearing loss: transforming silence to sound. *The Journal of Molecular Diagnostics*, *6*(4), 275-284.

Seeman, P., & Sakmaryová, I. (2006). High prevalence of the IVS 1 + 1 G to A/GJB2 mutation among Czech hearing impaired patients with monoallelic mutation in the coding region of GJB2. *Clinical Genetics*, *69*(5), 410-413.

Seeman, P., Bendová, O., Rasková, D., Malíková, M., Groh, D., & Kabelka, Z. (2005). Double Heterozygosity with Mutations Involving both the GJB2 and GJB6 Genes is a Possible, but very Rare, Cause of Congenital Deafness in the Czech Population. *Annals of Human Genetics*, *60*, 9-14.

Seeman, P., Malíková, M., Rasková, D., Bendová, O., Groh, D., Kubálková, M., Sakmaryová, I., *et al*. (2004). Spectrum and frequencies of mutations in the GJB2 (Cx26) gene among 156 Czech patients with pre-lingual deafness. *Clinical Genetics*, *66*(2), 152-157.

Seo, H. C., Kube, M., Edvardsen, R. B., Jensen, M. F., Beck, A., Spriet, E., Gorsky, G., *et al*. (2001). Miniature genome in the marine chordate Oikopleura dioica. *Science*, *294*(5551), 2506.

Shahin, H., Walsh, T., Sobe, T., Lynch, E., King, M.-C., Avraham, K. B., & Kanaan, M. (2002). Genetics of congenital deafness in the Palestinian population: multiple connexin 26 alleles with shared origins in the Middle East. *Human Genetics*, *110*(3), 284-289.

Shestopalov, V. I., & Panchin, Y. (2008). Pannexins and gap junction protein diversity. *Cellular and Molecular Life Sciences*, *65*(3), 376-394.

Shi, G.-Z., Gong, L.-X., Xu, X.-H., Nie, W.-Y., Lin, Q., & Qi, Y.-S. (2004). GJB2 gene mutations in newborns with non-syndromic hearing impairment in Northern China. *Hearing Research*, *197*(1-2), 19-23.

Shibata, T., Hibino, H., Doi, K., Suzuki, T., Hisa, Y., & Kurachi, Y. (2006). Gastric type H+,K+-ATPase in the cochlear lateral wall is critically involved in formation of the endocochlear potential. *American Journal of Physiology Cell Physiology*, *291*(5), C1038-C1048.

Sirmaci, A., Akcayoz-Duman, D., & Tekin, M. (2006). The c.IVS1+1G>A mutation in the GJB2 gene is prevalent and large deletions involving the GJB6 gene are not present in the Turkish population. *Journal of Genetics*, *85*(3), 213-216.

Skerrett, I. M., Di, W. L., Kasperek, E. M., Kelsell, D. P., & Nicholson, B. J. (2004). Aberrant gating, but a normal expression pattern, underlies the recessive phenotype of the deafness mutant Connexin26M34T. *The FASEB Journal*, *18*(7), 860-862.

Smith, R., & Van Camp, G. (1998). Nonsyndromic Hearing Loss and Deafness, DFNB1. In R. Pagon, T. Bird, C. Dolan, & K. Stephens (Eds.), *GeneReviews™ [Internet]*. Seattle (WA): University of Washington, Seattle.

Smith, R., Sheffield, A. M., & Van Camp, G. (1998). Nonsyndromic Hearing Loss and Deafness, DFNA3. In R. Pagon, T. Bird, C. Dolan, & K. Stephens (Eds.), *GeneReviews™ [Internet]*. Seattle (WA): University of Washington, Seattle.

Snoeckx, R. L., Hassan, D. M., Kamal, N. M., Van Den Bogaert, K., & Van Camp, G. (2005). Mutation analysis of the GJB2 (connexin 26) gene in Egypt. *Human Mutation*, *26*(1), 60-61.

Snoeckx, R. L., Huygen, P. L. M., Feldmann, D., Marlin, S., Denoyelle, F., Waligora, J., Mueller-Malesinska, M., *et al*. (2005). GJB2 mutations and degree of hearing loss: a multicenter study. *The American Journal of Human Genetics*, *77*(6), 945-957.

Sobe, T., Erlich, P., Berry, A., Korostichevsky, M., Vreugde, S., Avraham, K. B., Bonné-Tamir, B., *et al*. (1999). High frequency of the deafness-associated 167delT mutation in the connexin 26 (GJB2) gene in Israeli Ashkenazim. *American Journal of Medical Genetics*, *86*(5), 499-500.

Sodergren, E., Weinstock, G. M., Davidson, E. H., Cameron, R. A., Gibbs, R. A., Angerer, R. C., Angerer, L. M., *et al*. (2006). The genome of the sea urchin Strongylocentrotus purpuratus. *Science*, *314*(5801), 941-952.

Spicer, S. S., & Schulte, B. A. (1998). Evidence for a medial K+ recycling pathway from inner hair cells. *Hearing Research*, *118*(1-2), 1-12.

Stauffer, E. A., & Holt, J. R. (2007). Sensory transduction and adaptation in inner and outer hair cells of the mouse auditory system. *Journal of Neurophysiology*, *98*(6), 3360-3369.

Stenson, P. D., Mort, M., Ball, E. V., Howells, K., Phillips, A. D., Thomas, N. S., & Cooper, D. N. (2009). The Human Gene Mutation Database: 2008 update. *Genome Medicine*, *1*(1), 13.

Stong, B., Chang, Q., Ahmad, S., & Lin, X. (2006). A novel mechanism for connexin 26 mutation linked deafness: cell death caused by leaky gap junction hemichannels. *The Laryngoscope*, *116*(12), 2205-2210.

Sun, J., Ahmad, S., Chen, S., Tang, W., Zhang, Y., Chen, P., & Lin, X. (2005). Cochlear gap junctions coassembled from Cx26 and 30 show faster intercellular Ca2+ signaling than homomeric counterparts. *American Journal of Physiology Cell Physiology*, *288*(3), C613-C623.

Šterna, O., Proņina, N., Grīnfelde, I., Kušķe, S., Krūmiņa, A., Lugovska, R., & Pētersons, A. (2009). Spectrum and Frequency of the GJB2 Gene Mutations Among Latvian Patients with Prelingual Nonsyndromic Hearing Loss. *Proceedings of the Latvian Academy of Sciences Section B*, *63*(4/5 (663/664)), 198–203.

Taitelbaum-Swead, R., Brownstein, Z., Muchnik, C., Kishon-Rabin, L., Kronenberg, J., Megirov, L., Frydman, M., *et al*. (2006). Connexin-associated deafness and speech perception outcome of cochlear implantation. *Archives of Otolaryngology Head & Neck Surgery*, *132*(5), 495-500.

Tang, H.-Y., Fang, P., Ward, P. A., Schmitt, E., Darilek, S., Manolidis, S., Oghalai, J. S., *et al*. (2006). DNA sequence analysis of GJB2, encoding connexin 26: observations from a population of hearing impaired cases and variable carrier rates, complex genotypes, and ethnic stratification of alleles among controls. *American Journal of Medical Genetics Part A*, *140*(22), 2401-2415.

Teubner, B., Michel, V., Pesch, J., Lautermann, J., Cohen-Salmon, M., Söhl, G., Jahnke, K., *et al*. (2003). Connexin30 (Gjb6)-deficiency causes severe hearing impairment and lack of endocochlear potential. *Human Molecular Genetics*, *12*(1), 13-21.

The International HapMap Consortium. (n.d.). HapMap Data PhaseIII-Rel#3, May10, on NCBI B36 assembly, dbSNP b126: chr13:19562360.. 19762359. *International HapMap Project*. Retrieved July 17, 2010, from http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap3r3_B36/#search

Thierry-Mieg, D., & Thierry-Mieg, J. (2010). Homo sapiens gene GJB2, encoding gap junction protein, beta 2, 26kDa. *AceView: integrative annotation of cDNA-supported genes in human, mouse, rat, worm and Arabidopsis.* Retrieved April 13, 2011, from http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?db=human&term=GJB2

Thönnissen, E., Rabionet, R., Arbonès, M. L., Estivill, X., Willecke, K., & Ott, T. (2002). Human connexin26 (GJB2) deafness mutations affect the function of gap junction channels at different levels of protein expression. *Human Genetics*, *111*(2), 190-197.

Tóth, T., Kupka, S., Haack, B., Fazakas, F., Muszbek, L., Blin, N., Pfister, M., *et al*. (2007). Coincidence of mutations in different connexin genes in Hungarian patients. *International Journal of Molecular Medicine*, *20*(3), 315-321.

Unwin, P. N., & Zampighi, G. (1980). Structure of the junction between communicating cells. *Nature*, *283*(5747), 545-549.

Valiunas, V., Polosina, Y. Y., Miller, H., Potapova, I. A., Valiuniene, L., Doronin, S., Mathias, R. T., *et al*. (2005). Connexin-specific cell-to-cell transfer of short interfering RNA by gap junctions. *The Journal of Physiology*, *568*(Pt 2), 459-468.

Van Camp, G., & Smith, R. (2011a). Cochlea - blank. *Hereditary Hearing Loss Homepage*. Retrieved November 18, 2011, a from http://hereditaryhearingloss.org/main.aspx?c=.HHH&n=87131

Van Camp, G., & Smith, R. (2011b). Nonsyndromic Genes. *Hereditary Hearing Loss Homepage*. Retrieved December 1, 2011, b from http://hereditaryhearingloss.org/main.aspx?c=.HHH&n=86163

Wang, C. M., Lincoln, J., Cook, J. E., & Becker, D. L. (2007). Abnormal connexin expression underlies delayed wound healing in diabetic skin. *Diabetes*, *56*(11), 2809-2817.

Wang, H.-L., Chang, W.-T., Li, A. H., Yeh, T.-H., Wu, C.-Y., Chen, M.-S., & Huang, P.-C. (2003). Functional analysis of connexin-26 mutants associated with hereditary recessive deafness. *Journal of Neurochemistry*, *84*(4), 735-742.

Wangemann, P. (2002). K+ cycling and the endocochlear potential. *Hearing Research*, *165*(1-2), 1-9.

Wangemann, P. (2006). Supporting sensory transduction: cochlear fluid homeostasis and the endocochlear potential. *The Journal of Physiology*, *576*(Pt 1), 11-21.

White, T. W., Deans, M. R., Kelsell, D. P., & Paul, D. L. (1998). Connexin mutations in deafness. *Nature*, *394*(6694), 630-631.

White, T. W., Wang, H., Mui, R., Litteral, J., & Brink, P. R. (2004). Cloning and functional expression of invertebrate connexins from Halocynthia pyriformis. *FEBS Letters*, *577*(1-2), 42-8.

Wilch, E., Azaiez, H., Fisher, R. A., Elfenbein, J., Murgia, A., Birkenhäger, R., Bolz, H., *et al*. (2010). A novel DFNB1 deletion allele supports the existence of a distant cis-regulatory region that controls GJB2 and GJB6 expression. *Clinical Genetics*, *78*(3), 267-274.

Wilch, E., Zhu, M., Burkhart, K. B., Regier, M., Elfenbein, J. L., Fisher, R. A., & Friderici, K. H. (2006). Expression of GJB2 and GJB6 is reduced in a novel DFNB1 allele. *The American Journal of Human Genetics*, *79*(1), 174-179.

World Health Organization. (2011a). WHO | Deafness and hearing impairment. *WHO | World Health Organization*. World Health Organization. Retrieved May 11, 2011, a from http://www.who.int/mediacentre/factsheets/fs300/en/index.html

World Health Organization. (2011b). WHO | Grades of hearing impairment. *WHO | World Health Organization*. World Health Organization. Retrieved May 11, 2011, b from http://www.who.int/pbd/deafness/hearing_impairment_grades/en/index.html

Wu, B.-L., Lindeman, N., Lip, V., Adams, A., Amato, R. S., Cox, G., Irons, M., *et al*. (2002). Effectiveness of sequencing connexin 26 (GJB2) in cases of familial or sporadic childhood deafness referred for molecular diagnostic testing. *Genetics in Medicine*, *4*(4), 279-288.

Yeager, M. (1998). Structure of cardiac gap junction intercellular channels. *Journal of Structural Biology*, *121*(2), 231-245.

Yuan, Y., You, Y., Huang, D., Cui, J., Wang, Y., Wang, Q., Yu, F., *et al*. (2009). Comprehensive molecular etiology analysis of nonsyndromic hearing impairment from typical areas in China. *Journal of Translational Medicine*, *7*, 79.

Yuan, Y., Yu, F., Wang, G., Huang, S., Yu, R., Zhang, X., Huang, D., *et al*. (2010). Prevalence of the GJB2 IVS1+1G >A mutation in Chinese hearing loss patients with monoallelic pathogenic mutation in the coding region of GJB2. *Journal of Translational Medicine*, *8*, 127.

Zdebik, A. A., Wangemann, P., & Jentsch, T. J. (2009). Potassium ion movement in the inner ear: insights from genetic disease and mouse models. *Physiology*, *24*, 307-316.

Zhang, Y., Tang, W., Ahmad, S., Sipp, J. A., Chen, P., & Lin, X. (2005). Gap junction-mediated intercellular biochemical coupling in cochlear supporting cells is required for normal cochlear functions. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(42), 15201-15206.

Zhao, H.-B., & Yu, N. (2006). Distinct and gradient distributions of connexin26 and connexin30 in the cochlear sensory epithelium of guinea pigs. *Journal of Comparative Neurology*, *499*(3), 506-518.

Zidanic, M., & Brownell, W. E. (1990). Fine structure of the intracochlear potential field. I. The silent current. *Biophysical Journal*, *57*(6), 1253-1268.

Zoll, B., Petersen, L., Lange, K., Gabriel, P., Kiese-Himmel, C., Rausch, P., Berger, J., *et al*. (2003). Evaluation of Cx26/GJB2 in German hearing impaired persons: mutation spectrum and detection of disequilibrium between M34T (c.101T>C) and -493del10. *Human Mutation*, *21*(1), 98.

CHAPTER 2

GJB2 mutations and NSSHL in Portugal

CONTRIBUTION OF *GJB2* MUTATIONS TO NONSYNDROMIC SENSORINEURAL HEARING LOSS IN PORTUGAL

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ABSTRACT

Objective: A variable proportion of nonsyndromic sensorineural hearing loss (NSSHL) cases are due to mutations in the *GJB2* gene in several populations, which present distinct spectra and prevalence of such mutations. In order to assess the contribution of *GJB2* mutations to this pathology in the Portuguese population we have been investigating the prevalence of *GJB2* mutations in hearing impaired patients. We here present a first comprehensive molecular analysis focusing on 264 unrelated individuals (sporadic and familial cases) presenting with mild to profound bilateral NSSHL.

Methods: PCR-RFLP, SSCP and sequencing were used in the screening of the coding region, basal promoter, exon 1 and donor splice site of the *GJB2* gene. Multiplex PCR was used to screen for the two common *GJB6* deletions.

Results: At least one out of 21 different *GJB2* variants (18 coding and three noncoding) was identified in 80 (30.2%) of the 264 patients. Biallelic mutations were found in 53 (20%) of the probands, of which 83% (44/53) harboured at least one c.35delG allele. The most prevalent mutated genotype was [c.35delG]+[c.35delG], accounting for 52.8% (28/53) of the biallelic individuals. Next to this genotype, [c.35delG]+[p.Glu47X] (3/53 = 5.7%), [c.35delG]+[p.Trp172X] (3/53 = 5.7%), and [c.35delG]+[p.Val37Ile] (2/53 = 3.8%) were the most represented genotypes among bilallelic patients. Twenty-seven out of 264 (10.2%) probands were monoallelic, harbouring only one *GJB2* variant. Subsequent analysis revealed that the *GJB6* deletion del(*GJB6*-D13S1854) was present in at least 7.4% (2/27) of these monoallelic patients.

Conclusion: This study provides clear demonstration that mutations in the *GJB2* gene are an important cause of hearing impairment in Portugal. With basis solely on coding mutations, a diagnosis of *GJB2*-associated NSSHL was assumed for 18.9% (50/264) of the cases. Five other cases, with only one *GJB2* coding mutation, were elucidated by the identification of an additional DFNB1 mutation. Overall, 20.8% (55/264) of the patients were diagnosed as having DFNB1-related NSSHL, this representing a valuable indicator as regards therapeutical and rehabilitation options, as well as genetic counseling of these patients and their families.

INTRODUCTION

Hearing loss (HL) is the most common congenital sensory impairment, and the most prevalent sensorineural disorder (Hilgert, Smith, & Van Camp, 2009), being a major public health concern.

Regarding NSSHL, more than 140 genetic loci have been mapped, with more than 60 genes identified to date (Alford, 2011; Smith & Van Camp, 1998).

Despite this extreme genetic heterogeneity, mutations in the *GJB2* gene, encoding the protein connexin 26 (Cx26), are a major cause of NSSHL in many world populations, which makes *GJB2* the gene most frequently associated with this condition. Two gross deletions, del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854), involving the *GJB6* gene, which codes for connexin 30 (Cx30), have also been associated with nonsyndromic hearing loss (NSHL) in several cases, often in *trans* with a *GJB2* mutation (Lerer *et al*., 2001; Pallares-Ruiz, Blanchet, Mondain, Claustres, & Roux, 2002; F J del Castillo *et al*., 2005; I del Castillo *et al*., 2002). Both genes, *GJB2* and *GJB6*, are localised to chromosome region 13q11, corresponding to the DFNB1 locus.

Connexins associate in hexamers to form homo- or heteromeric connexons (Perkins, Goodenough, & Sosinsky, 1997; Yeager, 1998). Connexons of adjoining cells can bind forming intercellular channels which cluster into gap junctions (Perkins, Goodenough, & Sosinsky, 1998). Cx26 and Cx30 channels are believed to play a key role in the cycling of potassium (Kikuchi, Adams, Miyabe, So, & Kobayashi, 2000; Kikuchi, Kimura, Paul, & Adams, 1995; Kikuchi, Kimura, Paul, Takasaka, & Adams, 2000; Spicer & Schulte, 1998) which is critical for the normal sensory hair cell excitation. There is also evidence that cochlear gap junctions might be important for Ca^{2+} signaling (Sun *et al*., 2005) and the intracellular diffusion of large cationic molecules (Meşe, Valiunas, Brink, & White, 2008).

About two hundred *GJB2* mutations causing nonsyndromic HL have been reported (Stenson *et al*., 2009), most of them being recessive. A few specific mutations in *GJB2* also have been described in families with autosomal dominant HL (Smith, Sheffield, & Van Camp, 1998). Some mutations are very common and others are extremely rare. The carrier rate in the general

population for a recessive deafness causing *GJB2* mutation is approximately one in 33 (Smith, Shearer, Hildebrand, & Van Camp, 1999).

The spectrum and the prevalence of *GJB2* mutations vary among different populations. The recessive mutation c.35delG is the most frequent *GJB2* pathogenic mutation identified in NSHL European patients (Antoniadi *et al*., 2000; Gabriel *et al*., 2001; Janecke *et al*., 2002; Löppönen *et al*., 2003; Minarik, Ferakova, Ficek, Polakova, & Kadasi, 2005; Roux *et al*., 2004; Santos *et al*., 2005; Seeman *et al*., 2004; Šterna *et al*., 2009), in other populations of predominantly European ancestry (Batissoco *et al*., 2009; Dalamón *et al*., 2005; Kelley *et al*., 1998) and in the Mediterranean populations from Algeria (Ammar-Khodja *et al*., 2009), Egypt (Snoeckx, Hassan, Kamal, Van Den Bogaert, & Van Camp, 2005), Lebanon (Mustapha *et al*., 2001), Morocco (Abidi *et al*., 2007) and Tunisia (Masmoudi *et al*., 2000).

Three other recessive mutations, c.167delT, c.235delC, and p.Arg143Trp are the most frequent *GJB2* pathogenic mutations found among Ashkenazi Jews (Lerer *et al*., 2000), in Asian populations from Japan (Abe, Usami, Shinkawa, Kelley, & Kimberling, 2000; Fuse *et al*., 1999), Korea (H. J. Park, Hahn, Chun, Park, & Kim, 2000) and China (Dai *et al*., 2009), and in Ghanian populations (Brobby, Müller-Myhsok, & Horstmann, 1998; Hamelmann *et al*., 2001), respectively.

A different pathogenic mutation, p.Trp24X, was the predominant *GJB2* mutation identified in NSHL cases in India (RamShankar *et al*., 2003), and in Gypsies (an ethnic group that traces back to the Indian subcontinent) from Eastern Slovakia (Minárik *et al*., 2003) and Spain (Álvarez *et al*., 2005). This mutation was also found to have a high carrier frequency (2.4%) in the Indian population (RamShankar *et al*., 2003), and in some subpopulations of Gypsies (Minárik *et al*., 2003; Álvarez *et al*., 2005).

Due to the high frequency of *GJB2* mutations among NSHL cases in several populations worldwide, mutation analysis of this gene is a widely available genetic test, representing the first step in the molecular diagnosis of this pathology.

Since most *GJB2* mutations described so far localize to the *GJB2* coding region (totally included within exon 2), only this region has been systematically analysed. Nonetheless, a few noncoding pathogenic mutations have already been identified, contributing to the elucidation

of the genetic etiology of the HL in some patients who harboured only one recessive mutation in the *GJB2* coding region (Denoyelle *et al*., 1999; Mani *et al*., 2009; Matos *et al*., 2007).

In this study, we investigated the spectrum and prevalence of *GJB2* mutations in 264 Portuguese patients, and confirmed the relevance of screening this gene as a first step in the molecular analysis of NSSHL cases in our population.

MATERIALS AND METHODS

1. Patients

A total of 301 Portuguese unrelated individuals (sporadic and familial cases), presenting with mild to profound sensorineural hearing impairment were tested for the presence of mutations in the DFNB1 locus. They had been referred from various otolaryngology departments and genetic units. A recessive mode of inheritance was predominantly observed in the familial cases (at least one first- or second-degree affected relative).

In the analysis here presented, we only have considered those individuals affected with bilateral NSSHL. So, individuals with syndromic, unilateral, or acquired types of HL were excluded from this study. Diagnosis of NSSHL was established by accepted clinical criteria. Infection, oto-trauma or neonatal diseases causing acquired deafness were excluded by collecting detailed clinical history. Interestingly, there were three patients clinically diagnosed as having Waardenburg syndrome, and thus excluded from this study, who carried *GJB2*/*GJB6* mutations. One of them was a *GJB2*/*GJB6* compound heterozygous, with the [=]+[c.35delG]/[=]+[del(*GJB6*-D13S1854)] genotype, while the other two patients were heterozygous for the mutation p.Val153Ile in the *GJB2* gene. Individuals referred as not having been born in Portugal were also not considered. As such, of the 301 patients referred above, 264 were included in this analysis.

Written Informed consent was obtained from all the participants or from parents in case of those younger than 18 years.

2. Audiological methods

All patients involved in the study underwent otoscopic and audiometric examinations by using age-appropriate methods. Pure tone audiometry, tympanometry and auditory brainstem response were obtained in a sound proof room according to current clinical standards. We were not able to retrieve medical history and audiometric records for all the patients. For some patients family histories were unknown.

3. Molecular analysis

Blood samples were obtained from all the individuals and DNA was extracted using standard procedures.

All DNA samples were first screened for the 35delG mutation by PSDM-RFLP (Storm, Willocx, Flothmann, & Van Camp, 1999).

The 35delG homozygotes were diagnosed as having *GJB2*-related HL and, consequently, no other studies were performed.

The 35delG heterozygotes and the negative cases were further screened by single-strand conformational polymorphism (SSCP) analysis, and/or direct sequencing, of the entire coding region and flanking acceptor splice site of the *GJB2* gene, in order to detect whether other pathogenic/controversial *GJB2* mutations were present.

If the cause of deafness remained non-elucidated, the patients were then screened for the del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) *GJB6* deletions using multiplex PCR, according to F J del Castillo *et al*. (2005) and I del Castillo *et al*. (2002). When obtaining no positive result, a number of patients were also screened by sequencing for mutations in the *GJB2* basal promoter, exon 1 and donor splice site (most of these patients have been described in Matos *et al*. (2008, 2007, 2011, 2010).

Some patients could not be analysed regarding the *GJB6* deletions and/or the noncoding regions of *GJB2*.

RESULTS

At least one out of 21 different *GJB2* variants (18 coding and three noncoding) was found in 80 (30.2%) of the 264 patients (see Table 1). These include 12 missense, 3 nonsense and 2 frameshift variants, 1 in frame deletion, 1 mutation in the donor splice site, 1 mutation in the basal promoter, and 1 polymorphism in the intron. All the variants here identified were previously described, with the exception of the novel variant c.24G>A, which results in a silent mutation at protein level (p.Thr8Thr). Two of the reported mutations (c.-259C>T and p.Met163Leu), listed in Table 1, were first found in Portuguese patients (Matos *et al*., 2008, 2007) belonging to the sample under analysis. As expected, c.35delG was the mutation most frequently found amongst the NSSHL patients, being present in 84 of the 528 chromosomes analysed, which corresponds to a prevalence of 15.9% (see Table 1).

Table 2 shows the frequencies of *GJB2* genotypes identified in a total of 264 individuals presenting with bilateral NSSHL. *GJB2* biallelic mutations were found in 53 (20%) of the probands, of which 83% (44/53) harboured at least one c.35delG allele. The most prevalent mutated genotype was [c.35delG]+[c.35delG], accounting for 52.8% (28/53) of the biallelic individuals. Next to this genotype, [c.35delG]+[p.Glu47X] (3/53 = 5.7%), [c.35delG]+[p.Trp172X] (3/53 = 5.7%), and [c.35delG]+[p.Val37Ile] (2/53 = 3.8%) genotypes were the most represented among bilallelic patients.

Twenty-seven out of 264 (10.2%) probands were monoallelic, harbouring only one *GJB2* variant. Subsequent analysis revealed that the *GJB6* deletion del(*GJB6*-D13S1854) was present in two of those patients (one heterozygous for c.35delG plus one heterozygous for p.Met34Thr), presenting with prelingual, profound HL, which was then considered to be due to their *GJB2*/*GJB6* compound genotype. In one monoallelic patient, harbouring the dominant p.Met163Leu mutation, the *GJB2* genotype was shown to be responsible for the HL (this patient and her mother were described in Matos *et al*. (2008).

DISCUSSION

A wide spectrum of *GJB2* pathogenic, unclear or controversial mutations was observed in the cohort of 264 NSSHL Portuguese individuals included in this study, among which two variants so far found in these patients only, c.-259C>T, of confirmed pathogenicity, and p.Thr8Thr, with unknown effects.

The DFNB1 genotype explained the HL, or was a likely cause of the pathology, in 20.8% (55/264) of the cases. All but two of these cases (53/55), corresponding to 1 in 5 of the patients analysed, were due to a *GJB2* genotype. The *GJB2*-related cases include one monoallelic patient harbouring the dominant p.Met163Leu mutation. The HL in three patients with one coding *GJB2* mutation was explained by the presence of a second *GJB2* mutation, in the basal promoter or in the donor splice site, highlighting the relevance of screening those noncoding regions in addition to the coding region.

The deletion c.35delG was the predominant *GJB2* mutation among *GJB2*-related HL cases, in accordance with other Caucasian or of mainly Caucasian descent populations (Dalamón *et al*., 2010; Pandya *et al*., 2003; Roux *et al*., 2004). Individuals homozygous for the c.35delG mutation accounted for about 53% of the *GJB2*-related HL cases, and represented 10.6% of the total Portuguese NSSHL patients.

Interestingly, an excess of c.35delG heterozygotes (11/264) was observed. Their allelic frequency corresponds to 4.1%, which is very high when compared with the c.35delG carrier rate in the Portuguese general population (0.88%) (data not yet published). It should be noted that we were not able to analyse all the 11 patients for the *GJB6* deletions and the *GJB2* noncoding regions considered in this study. So, although some of these individuals might just be coincidental carriers, some other patients may harbour a second DFNB1 mutation, even in other *GJB2*/*GJB6* regions not here analysed, thus explaining the excess of c.35delG heterozygotes.

As regards genotype-phenotype correlations, truncating (T) mutations, as is the case of c.35delG, are usually associated with a greater degree of HL than nontruncating (NT) mutations (Cryns *et al*., 2004; Putcha *et al*., 2007; Snoeckx, Huygen, *et al*., 2005). However, variability in the degree of the hearing loss in T/T genotypes has been noticed, and is well exemplified by the

significant intra- and inter-familial variation, ranging from mild to profound impairment, which has been observed in c.35delG homozygotes (Cryns *et al*., 2004; Murgia *et al*., 1999). In the present study, the majority (16) of homozygotes for c.35delG had severe or profound HL, as expected. However, in five of the homozygous patients the hearing impairment was moderate.

As opposed to T/T *GJB2* genotypes, NT/NT *GJB2* genotypes appear to be often associated with mild or moderate HL (Putcha *et al*., 2007; Snoeckx, Huygen, *et al*., 2005). As regards NT/NT genotypes including at least one p.Met34Thr or p.Val37Ile allele, it was observed that they were usually, but not always, associated with less severe HL (Snoeckx, Huygen, *et al*., 2005). Noteworthy, in our sample, two sporadic cases were compound heterozygous for p.Val37Ile plus one of two other NT mutations, p.Arg184Trp and p.Asn206Ser, and both presented with severe HL.

In which concerns T/NT *GJB2* genotypes, the variability on HL degree seems to depend on the NT mutation, at least when the T mutation is c.35delG (an early frameshift mutation). The two patients who were compound heterozygous for the p.Val37Ile and c.35delG displayed mild and moderate HL, respectively, which is in accordance with the phenotypes previously found associated with this compound genotype by Cryns *et al*. (2004). The association of the [c.35delG]+[p.Val37Ile] genotype with less severe HL phenotypes was corroborated by Pollak *et al*. (2007) and Snoeckx, Huygen, *et al*. (2005). One other T/NT *GJB2* genotype, [c.35delG]+[p.Leu90Pro], is most commonly associated with mild or moderate HL (Cryns *et al*., 2004; Pollak *et al*., 2007; Snoeckx, Huygen, *et al*., 2005). Our patient presenting this genotype had a moderate hearing impairment. As regards the genotype [c.35delG]+[p.Asn206Ser], it apparently follows the same trend as the [c.35delG]+[p.Leu90Pro] genotype (Marlin *et al*., 2005; Snoeckx, Huygen, *et al*., 2005). The [c.35delG]+[p.Asn206Ser] patient here reported presented with moderate HL. On the contrary, the [c.35delG]+[p.Val95Met] genotype, here found in two patients with severe HL, effectively appears to be more often associated with severe to profound HL (Cryns *et al*., 2004; Snoeckx, Huygen, *et al*., 2005) than with less severe HL (Cheng *et al*., 2005).

Interestingly, the dominant *GJB2* mutation p.Met163Leu, causing mild/moderate high-frequency HL (Matos *et al*., 2008) has meanwhile also been found in one Argentinean patient

presenting with prelingual, profound NSSHL (Dalamón *et al*., 2010), suggesting that the severity of the HL due to the p.Met163Leu mutation might be variable and/or progressive.

The pathogenicity of four *GJB2* coding mutations found in our sample has been regarded as unclear or controversial in different studies. These mutations are p.Met34Thr (Bicego *et al*., 2006; Cama *et al*., 2009; Feldmann *et al*., 2004; Griffith *et al*., 2000; Kenna, Wu, Cotanche, Korf, & Rehm, 2001; Pollak *et al*., 2007; Snoeckx, Huygen, *et al*., 2005; Wu *et al*., 2002), p.Arg127His (Matos *et al*., 2010), p.Val153Ile (Cryns *et al*., 2004; Dalamón *et al*., 2005; Hashemzadeh Chaleshtori *et al*., 2005; Kenna *et al*., 2001; Marlin *et al*., 2001; RamShankar *et al*., 2003; Snoeckx, Hassan, *et al*., 2005; Snoeckx, Huygen, *et al*., 2005) and p.Gly160Ser (Cheng *et al*., 2005; Hashemzadeh Chaleshtori, Farhud, & Patton, 2007; Janecke *et al*., 2002; Löffler *et al*., 2001; Santos *et al*., 2005; Snoeckx, Huygen, *et al*., 2005; Tang *et al*., 2006).

In our study, p.Val153Ile and p.Gly160Ser were the sole *GJB2* mutations identified in two individuals with mild and moderate hearing impairment, respectively. These two patients were also analysed in respect to both *GJB6* deletions and the basal promoter, exon 1 and donor splice site of *GJB2* gene, and no mutation was identified. So, no conclusion could be drawn. On the contrary, mutations p.Met34Thr and p.Arg127His were identified in biallelic patients, whose genotype we have considered to be the likely cause of the HL, with some reservations regarding the only p.Arg127His homozygous patient. Effectively, the [p.Arg127His]+[p.Arg127His] genotype was found segregating with HL (Tóth *et al*., 2004) but it was also identified in normal hearing individuals in the Indian population (RamShankar *et al*., 2003). Also, in one of the families included in this study, the p.Arg127His homozygous genotype is not segregating with HL (Matos *et al*., 2010). As regards the referred p.Arg127His homozygous patient, he doesn't harbour any of the *GJB6* deletions. Since other hearing impaired relatives of this patient were not available for study, we cannot know whether the p.Arg127His homozygous genotype is segregating with HL in the family.

In general, our data meet the view that severity of *GJB2*-related HL can be correlated with specific mutation combinations (Cryns *et al*., 2004; Snoeckx, Huygen, *et al*., 2005). Variation in the phenotype for a given genotype would likely be dependent on more than one major

modifier gene, as suggested by the study of Hilgert, Huentelman, *et al*. (2009), as well as on environmental factors.

Finally, our study is suggestive of genetic influx from other populations, since we found in the Portuguese patients some mutations particularly frequent in certain populations: c.-23+1G>A (Eastern Europeans, Turkish, and Kurdish population of Iran), p.Trp24X and p.Arg127His (Indians/Gypsies), p.Val37Ile (East Asians), c.167delT (Ashkenazi Jews), c.-22-12C>T (Sub-Saharan Africans). It should be noted that the patients in this study harbouring p.Trp24X mutation are of Gypsy or Indian ethnicity/ancestry. This fact may account for the observed high frequency of this mutation.

CONCLUSION

GJB2 mutations are an important cause of NSSHL in the Portuguese population, being c.35delG the predominant mutation, with each of the 19 other pathogenic/controversial variants occurring at much lower frequencies.

With basis solely on coding mutations, a diagnosis of *GJB2*-associated hearing loss was confirmed for 18.9% of the cases. Screening for the *GJB2* donor splice site and the basal promoter, as well as for the two common *GJB6* deletions, allowed the elucidation of the etiology of the HL in some of the cases with only one coding mutation identified. Therefore, such screening, in monoallelic patients, is advisable and should be performed.

Overall, 20.8% of the patients were diagnosed as having DFNB1-related NSSHL, which represents a valuable indicator as regards therapeutical and rehabilitation options, as well as genetic counseling of Portuguese patients and their families.

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REFERENCES

Abe, S., Usami, S., Shinkawa, H., Kelley, P., & Kimberling, W. (2000). Prevalent connexin 26 gene (GJB2) mutations in Japanese. *Journal of Medical Genetics*, *37*(1), 41-43.

Abidi, O., Boulouiz, R., Nahili, H., Ridal, M., Alami, M. N., Tlili, A., Rouba, H., *et al*. (2007). GJB2 (connexin 26) gene mutations in Moroccan patients with autosomal recessive non-syndromic hearing loss and carrier frequency of the common GJB2-35delG mutation. *International Journal of Pediatric Otorhinolaryngology*, *71*(8), 1239-1245.

Alford, R. (2011). Nonsyndromic Hereditary Hearing Loss. In R. Alford & V. Sutton (Eds.), *Medical Genetics in the Clinical Practice of ORL* (pp. 37-42).

Ammar-Khodja, F., Faugère, V., Baux, D., Giannesini, C., Léonard, S., Makrelouf, M., Malek, R., *et al*. (2009). Molecular screening of deafness in Algeria: high genetic heterogeneity involving DFNB1 and the Usher loci, DFNB2/USH1B, DFNB12/USH1D and DFNB23/USH1F. *European Journal of Medical Genetics*, *52*(4), 174-179.

Antoniadi, T., Gronskov, K., Sand, A., Pampanos, A., Brondum-Nielsen, K., & Petersen, M. B. (2000). Mutation analysis of the GJB2 (connexin 26) gene by DGGE in Greek patients with sensorineural deafness. *Human Mutation*, *16*(1), 7-12.

Álvarez, A., Del Castillo, I., Villamar, M., Aguirre, L. A., González-Neira, A., López-Nevot, A., Moreno-Pelayo, M. A., *et al*. (2005). High prevalence of the W24X mutation in the gene encoding connexin-26 (GJB2) in Spanish Romani (gypsies) with autosomal recessive non-syndromic hearing loss. *American Journal of Medical Genetics Part A*, *137A*(3), 255-258.

Batissoco, A. C., Abreu-Silva, R. S., Braga, M. C. C., Lezirovitz, K., Della-Rosa, V., Alfredo, T., Otto, P. A., *et al*. (2009). Prevalence of GJB2 (connexin-26) and GJB6 (connexin-30) mutations in a cohort of 300 Brazilian hearing-impaired individuals: implications for diagnosis and genetic counseling. *Ear and Hearing*, *30*(1), 1-7.

Bicego, M., Beltramello, M., Melchionda, S., Carella, M., Piazza, V., Zelante, L., Bukauskas, F. F., *et al*. (2006). Pathogenetic role of the deafness-related M34T mutation of Cx26. *Human Molecular Genetics*, *15*(17), 2569-2587.

Brobby, G. W., Müller-Myhsok, B., & Horstmann, R. D. (1998). Connexin 26 R143W mutation associated with recessive nonsyndromic sensorineural deafness in Africa. *The New England Journal of Medicine*, *338*(8), 548-550.

Cama, E., Melchionda, S., Palladino, T., Carella, M., Santarelli, R., Genovese, E., Benettazzo, F., *et al*. (2009). Hearing loss features in GJB2 biallelic mutations and GJB2/GJB6 digenic inheritance in a large Italian cohort. *International Journal of Audiology*, *48*(1), 12-17.

Cheng, X., Li, L., Brashears, S., Morlet, T., Ng, S. S., Berlin, C., Hood, L., *et al*. (2005). Connexin 26 variants and auditory neuropathy/dys-synchrony among children in schools for the deaf. *American Journal of Medical Genetics Part A*, *139*(1), 13-18.

Cryns, K., Orzan, E., Murgia, A., Huygen, P., Moreno, F., Del Castillo, I., Parker, C., *et al*. (2004). A genotype-phenotype correlation for GJB2 (connexin 26) deafness. *Journal of Medical Genetics*, *41*(3), 147-154.

Dai, P., Yu, F., Han, B., Liu, X., Wang, G., Li, Q., Yuan, Y., *et al*. (2009). GJB2 mutation spectrum in 2063 Chinese patients with nonsyndromic hearing impairment. *Journal of Translational Medicine*, *7*(April), 26.

Dalamón, V., Béhèran, A., Diamante, F., Pallares, N., Diamante, V., & Elgoyhen, A. B. (2005). Prevalence of GJB2 mutations and the del (GJB6-D13S1830) in Argentinean non-syndromic deaf patients. *Hearing Research*, *207*, 43-49.

Dalamón, V., Lotersztein, V., Béhèran, A., Lipovsek, M., Diamante, F., Pallares, N., Francipane, L., *et al*. (2010). GJB2 and GJB6 genes: molecular study and identification of novel GJB2 mutations in the hearing-impaired Argentinean population. *Audiology & Neurotology*, *15*(3), 194-202.

del Castillo, F. J., Rodriguez-Ballest, M., Alvarez, A., Hutchin, T., Leonardi, E., de Oliveira, C. A., Azaiez, H., *et al*. (2005). A novel deletion involving the connexin-30 gene, del(GJB6-d13s1854), found in trans with mutations in the GJB2 gene (connexin-26) in subjects with DFNB1 non-syndromic hearing impairment. *Journal of Medical Genetics*, *42*(7), 588-594.

del Castillo, I., Villamar, M., Moreno-Pelayo, M. A., del Castillo, F. J., Alvarez, A., Tellería, D., Menéndez, I., *et al*. (2002). A deletion involving the connexin 30 gene in nonsyndromic hearing impairment. *The New England Journal of Medicine*, *346*(4), 243-249.

Denoyelle, F., Marlin, S., Weil, D., Moatti, L., Chauvin, P., Garabédian, E. N., & Petit, C. (1999). Clinical features of the prevalent form of childhood deafness, DFNB1, due to a connexin-26 gene defect: implications for genetic counselling. *The Lancet*, *353*(9161), 1298-1303.

Feldmann, D., Denoyelle, F., Loundon, N., Weil, D., Garabedian, E.-N., Couderc, R., Joannard, A., *et al*. (2004). Clinical evidence of the nonpathogenic nature of the M34T variant in the connexin 26 gene. *European Journal of Human Genetics*, *12*(4), 279-284.

Fuse, Y., Doi, K., Hasegawa, T., Sugii, A., Hibino, H., & Kubo, T. (1999). Three novel connexin26 gene mutations in autosomal recessive non-syndromic deafness. *NeuroReport*, *10*(9), 1853-1857.

Gabriel, H., Kupsch, P., Sudendey, J., Winterhager, E., Jahnke, K., & Lautermann, J. (2001). Mutations in the connexin26/GJB2 gene are the most common event in non-syndromic hearing loss among the German population. *Human Mutation*, *17*(6), 521-522.

Griffith, A. J., Chowdhry, A. A., Kurima, K., Hood, L. J., Keats, B., Berlin, C. I., Morell, R. J., *et al*. (2000). Autosomal recessive nonsyndromic neurosensory deafness at DFNB1 not associated with the compound-heterozygous GJB2 (connexin 26) genotype M34T/167delT. *The American Journal of Human Genetics*, *67*(3), 745-749.

Hamelmann, C., Amedofu, G. K., Albrecht, K., Muntau, B., Brobby, G. W., & Horstmann, R. D. (2001). Pattern of Connexin 26 (GJB2) Mutations Causing Sensorineural Hearing Impairment in Ghana. *Blood*, *428*(March), 1-6.

Hashemzadeh Chaleshtori, M., Farhud, D. D., & Patton, M. A. (2007). Familial and Sporadic GJB2-Related Deafness in Iran: Review of Gene Mutations. *Iranian Journal of Public Health*, *36*(1), 1-14.

Hashemzadeh Chaleshtori, M., Rad, L. H., Dolati, M., Sasanfar, R., Hoseinipour, A., Zohour, M. M., Pourjafari, H., *et al*. (2005). Frequencies of Mutations in the Connexin 26 Gene (GJB2) in Two Populations of Iran (Tehran and Tabriz). *Iranian Journal of Public Health*, *34*(1), 1-7.

Hilgert, N., Huentelman, M. J., Thorburn, A. Q., Fransen, E., Dieltjens, N., Mueller-Malesinska, M., Pollak, A., *et al*. (2009). Phenotypic variability of patients homozygous for the GJB2 mutation 35delG cannot be explained by the influence of one major modifier gene. *European Journal of Human Genetics*, *17*(4), 517-524.

Hilgert, N., Smith, R. J., & Van Camp, G. (2009). Forty-six genes causing nonsyndromic hearing impairment: which ones should be analyzed in DNA diagnostics? *Mutation Research*, *681*(2-3), 189-196.

Janecke, A. R., Hirst-Stadlmann, A., Günther, B., Utermann, B., Müller, T., Löffler, J., Utermann, G., *et al*. (2002). Progressive hearing loss, and recurrent sudden sensorineural hearing loss associated with GJB2 mutations--phenotypic spectrum and frequencies of GJB2 mutations in Austria. *Human Genetics*, *111*(2), 145-153.

Kelley, P. M., Harris, D. J., Comer, B. C., Askew, J. W., Fowler, T., Smith, S. D., & Kimberling, W. J. (1998). Novel mutations in the connexin 26 gene (GJB2) that cause autosomal recessive (DFNB1) hearing loss. *The American Journal of Human Genetics*, *62*(4), 792-799.

Kenna, M. A., Wu, B. L., Cotanche, D. A., Korf, B. R., & Rehm, H. L. (2001). Connexin 26 studies in patients with sensorineural hearing loss. *Archives of Otolaryngology Head & Neck Surgery*, *127*(9), 1037-1042.

Kikuchi, T., Adams, J. C., Miyabe, Y., So, E., & Kobayashi, T. (2000). Potassium ion recycling pathway via gap junction systems in the mammalian cochlea and its interruption in hereditary nonsyndromic deafness. *Medical Electron Microscopy*, *33*(2), 51-56.

Kikuchi, T., Kimura, R. S., Paul, D. L., & Adams, J. C. (1995). Gap junctions in the rat cochlea: immunohistochemical and ultrastructural analysis. *Anatomy and Embryology*, *191*(2), 101-118.

Kikuchi, T., Kimura, R. S., Paul, D. L., Takasaka, T., & Adams, J. C. (2000). Gap junction systems in the mammalian cochlea. *Brain Research Brain Research Reviews*, *32*(1), 163-166.

Lerer, I., Sagi, M., Ben-Neriah, Z., Wang, T., Levi, H., & Abeliovich, D. (2001). A deletion mutation in GJB6 cooperating with a GJB2 mutation in trans in non-syndromic deafness: A novel founder mutation in Ashkenazi Jews. *Human Mutation*, *18*(5), 460.

Lerer, I., Sagi, M., Malamud, E., Levi, H., Raas-Rothschild, A., & Abeliovich, D. (2000). Contribution of connexin 26 mutations to nonsyndromic deafness in Ashkenazi patients and the variable phenotypic effect of the mutation 167delT. *American Journal of Medical Genetics*, *95*(1), 53-56.

Löffler, J., Nekahm, D., Hirst-Stadlmann, A., Günther, B., Menzel, H. J., Utermann, G., & Janecke, A. R. (2001). Sensorineural hearing loss and the incidence of Cx26 mutations in Austria. *European Journal of Human Genetics*, *9*(3), 226-230.

Löppönen, T., Väisänen, M.-L., Luotonen, M., Allinen, M., Uusimaa, J., Lindholm, P., Mäki-Torkko, E., *et al*. (2003). Connexin 26 mutations and nonsyndromic hearing impairment in northern Finland. *The Laryngoscope*, *113*(10), 1758-1763.

Mani, R. S., Ganapathy, A., Jalvi, R., Srikumari Srisailapathy, C. R., Malhotra, V., Chadha, S., Agarwal, A., *et al*. (2009). Functional consequences of novel connexin 26 mutations associated with hereditary hearing loss. *European Journal of Human Genetics*, *17*(4), 502-509.

Marlin, S., Feldmann, D., Blons, H., Loundon, N., Rouillon, I., Albert, S., Chauvin, P., *et al*. (2005). GJB2 and GJB6 mutations: genotypic and phenotypic correlations in a large cohort of hearing-impaired patients. *Archives of Otolaryngology Head & Neck Surgery*, *131*(6), 481-487.

Marlin, S., Garabédian, E. N., Roger, G., Moatti, L., Matha, N., Lewin, P., Petit, C., *et al*. (2001). Connexin 26 gene mutations in congenitally deaf children: pitfalls for genetic counseling. *Archives of Otolaryngology Head & Neck Surgery*, *127*(8), 927-933.

Masmoudi, S., Elgaied-Boulila, A., Kassab, I., Arab, S. B., Blanchard, S., Bouzoita, J., Drira, M., *et al*. (2000). Determination of the frequency of connexin26 mutations in inherited sensorineural deafness and carrier rates in the Tunisian population using DGGE. *Journal of Medical Genetics*, *37*(11), e39.

Matos, T. D., Caria, H., Simões-Teixeira, H., Aasen, T., Dias, O., Andrea, M., Kelsell, D. P., *et al*. (2008). A novel M163L mutation in connexin 26 causing cell death and associated with autosomal dominant hearing loss. *Hearing Research*, *240*(1-2), 87-92.

Matos, T. D., Caria, H., Simões-Teixeira, H., Aasen, T., Nickel, R., Jagger, D. J., O'Neill, A., *et al*. (2007). A novel hearing-loss-related mutation occurring in the GJB2 basal promoter. *Journal of Medical Genetics*, *44*(11), 721-725.

Matos, T. D., Simões-Teixeira, H., Caria, H., Cascão, R., Rosa, H., O'Neill, A., Dias, Ó., *et al*. (2011). Assessing Noncoding Sequence Variants of GJB2 for Hearing Loss Association. *Genetics Research International*, *2011*. doi:10.4061/2011/827469

Matos, T. D., Simões-Teixeira, H., Caria, H., Rosa, H., O'Neill, A., & Fialho, G. (2010). The controversial p.Arg127His mutation in GJB2: report on three Portuguese hearing loss family cases. *Genetic Testing and Molecular Biomarkers*, *14*(1), 141-144.

Meşe, G., Valiunas, V., Brink, P. R., & White, T. W. (2008). Connexin26 deafness associated mutations show altered permeability to large cationic molecules. *American Journal of Physiology Cell Physiology*, *295*(4), C966-C974.

Minarik, G., Ferakova, E., Ficek, A., Polakova, H., & Kadasi, L. (2005). GJB2 gene mutations in Slovak hearing-impaired patients of Caucasian origin: spectrum, frequencies and SNP analysis. *Clinical Genetics*, *68*(6), 554-557.

Minárik, G., Ferák, V., Feráková, E., Ficek, A., Poláková, H., & Kádasi, L. (2003). High frequency of GJB2 mutation W24X among Slovak Romany (Gypsy) patients with non-syndromic hearing loss (NSHL). *General Physiology and Biophysics*, *22*(4), 549-556.

Murgia, A., Orzan, E., Polli, R., Martella, M., Vinanzi, C., Leonardi, E., Arslan, E., *et al*. (1999). Cx26 deafness: mutation analysis and clinical variability. *Journal of Medical Genetics*, *36*(11), 829-832.

Mustapha, M., Salem, N., Delague, V., Chouery, E., Ghassibeh, M., Rai, M., Loiselet, J., *et al*. (2001). Autosomal recessive non-syndromic hearing loss in the Lebanese population: prevalence of the 30delG mutation and report of two novel mutations in the connexin 26 (GJB2) gene. *Journal of Medical Genetics*, *38*(10), e36.

Pallares-Ruiz, N., Blanchet, P., Mondain, M., Claustres, M., & Roux, A.-F. (2002). A large deletion including most of GJB6 in recessive non syndromic deafness: a digenic effect? *European Journal of Human Genetics*, *10*(1), 72-76.

Pandya, A., Arnos, K. S., Xia, X. J., Welch, K. O., Blanton, S. H., Friedman, T. B., Garcia Sanchez, G., *et al*. (2003). Frequency and distribution of GJB2 (connexin 26) and GJB6 (connexin 30) mutations in a large North American repository of deaf probands. *Genetics in Medicine*, *5*(4), 295-303.

Park, H. J., Hahn, S. H., Chun, Y. M., Park, K., & Kim, H. N. (2000). Connexin26 mutations associated with nonsyndromic hearing loss. *The Laryngoscope*, *110*(9), 1535-1538.

Perkins, G., Goodenough, D., & Sosinsky, G. (1997). Three-dimensional structure of the gap junction connexon. *Biophysical Journal*, *72*(2 Pt 1), 533-544.

Perkins, G., Goodenough, D., & Sosinsky, G. (1998). Formation of the gap junction intercellular channel requires a 30 degree rotation for interdigitating two apposing connexons. *Journal of Molecular Biology*, *277*(2), 171-177.

Pollak, A., Skórka, A., Mueller-Malesińska, M., Kostrzewa, G., Kisiel, B., Waligóra, J., Krajewski, P., *et al*. (2007). M34T and V37I mutations in GJB2 associated hearing impairment: evidence for pathogenicity and reduced penetrance. *American Journal of Medical Genetics Part A*, *143A*(21), 2534-2543.

Putcha, G. V., Bejjani, B. A., Bleoo, S., Booker, J. K., Carey, J. C., Carson, N., Das, S., *et al*. (2007). A multicenter study of the frequency and distribution of GJB2 and GJB6 mutations in a large North American cohort. *Genetics in Medicine*, *9*(7), 413-426.

RamShankar, M., Girirajan, S., Dagan, O., Ravi, S., Jalvi, R., Rangasayee, R., Avraham, K., *et al*. (2003). Contribution of connexin26 (GJB2) mutations and founder effect to non-syndromic hearing loss in India. *Journal of Medical Genetics*, *40*(5), e68.

Roux, A.-F., Pallares-Ruiz, N., Vielle, A., Faugère, V., Templin, C., Leprevost, D., Artières, F., *et al*. (2004). Molecular epidemiology of DFNB1 deafness in France. *BMC Medical Genetics*, *5*, 5.

Santos, R. L. P., Aulchenko, Y. S., Huygen, P. L. M., Van Der Donk, K. P., De Wijs, I. J., Kemperman, M. H., Admiraal, R. J. C., *et al*. (2005). Hearing impairment in Dutch patients with connexin 26 (GJB2) and connexin 30 (GJB6) mutations. *International Journal of Pediatric Otorhinolaryngology*, *69*(2), 165-174.

Seeman, P., Malíková, M., Rasková, D., Bendová, O., Groh, D., Kubálková, M., Sakmaryová, I., *et al*. (2004). Spectrum and frequencies of mutations in the GJB2 (Cx26) gene among 156 Czech patients with pre-lingual deafness. *Clinical Genetics*, *66*(2), 152-157.

Smith, R. J., & Van Camp, G. (1998). Nonsyndromic Hearing Loss and Deafness, DFNB1. In R. Pagon, T. Bird, C. Dolan, & K. Stephens (Eds.), *GeneReviews™ [Internet]*. Seattle (WA): University of Washington, Seattle.

Smith, R. J., Shearer, A. E., Hildebrand, M. S., & Van Camp, G. (1999). Deafness and Hereditary Hearing Loss Overview. In R. Pagon, T. Bird, C. Dolan, & K. Stephens (Eds.), *GeneReviews™ [Internet]*. Seattle (WA): University of Washington, Seattle.

Smith, R. J., Sheffield, A. M., & Van Camp, G. (1998). Nonsyndromic Hearing Loss and Deafness, DFNA3. In R. Pagon, T. Bird, C. Dolan, & K. Stephens (Eds.), *GeneReviews™ [Internet]*. Seattle (WA): University of Washington, Seattle.

Snoeckx, R. L., Hassan, D. M., Kamal, N. M., Van Den Bogaert, K., & Van Camp, G. (2005). Mutation analysis of the GJB2 (connexin 26) gene in Egypt. *Human Mutation*, *26*(1), 60-61.

Snoeckx, R. L., Huygen, P. L. M., Feldmann, D., Marlin, S., Denoyelle, F., Waligora, J., Mueller-Malesinska, M., *et al*. (2005). GJB2 mutations and degree of hearing loss: a multicenter study. *The American Journal of Human Genetics*, *77*(6), 945-957.

Spicer, S. S., & Schulte, B. A. (1998). Evidence for a medial K^+ recycling pathway from inner hair cells. *Hearing Research*, *118*(1-2), 1-12.

Stenson, P. D., Mort, M., Ball, E. V., Howells, K., Phillips, A. D., Thomas, N. S., & Cooper, D. N. (2009). The Human Gene Mutation Database: 2008 update. *Genome Medicine*, *1*(1), 13.

Storm, K., Willocx, S., Flothmann, K., & Van Camp, G. (1999). Determination of the carrier frequency of the common GJB2 (connexin-26) 35delG mutation in the Belgian population using an easy and reliable screening method. *Human Mutation*, *14*(3), 263-266.

Sun, J., Ahmad, S., Chen, S., Tang, W., Zhang, Y., Chen, P., & Lin, X. (2005). Cochlear gap junctions coassembled from Cx26 and 30 show faster intercellular Ca^{2+} signaling than homomeric counterparts. *American Journal of Physiology Cell Physiology*, *288*(3), C613-C623.

Šterna, O., Proņina, N., Grīnfelde, I., Kušķe, S., Krūmiņa, A., Lugovska, R., & Pētersons, A. (2009). Spectrum and Frequency of the GJB2 Gene Mutations Among Latvian Patients with Prelingual Nonsyndromic Hearing Loss. *Proceedings of the Latvian Academy of Sciences Section B*, *63*(4/5 (663/664)), 198–203.

Tang, H.-Y., Fang, P., Ward, P. A., Schmitt, E., Darilek, S., Manolidis, S., Oghalai, J. S., *et al*. (2006). DNA sequence analysis of GJB2, encoding connexin 26: observations from a population of hearing impaired cases and variable carrier rates, complex genotypes, and ethnic stratification of alleles among controls. *American Journal of Medical Genetics Part A*, *140*(22), 2401-2415.

Tóth, T., Kupka, S., Haack, B., Riemann, K., Braun, S., Fazakas, F., Zenner, H.-P., *et al*. (2004). GJB2 mutations in patients with non-syndromic hearing loss from Northeastern Hungary. *Human Mutation*, *23*(6), 631-632.

Wu, B.-L., Lindeman, N., Lip, V., Adams, A., Amato, R. S., Cox, G., Irons, M., *et al*. (2002). Effectiveness of sequencing connexin 26 (GJB2) in cases of familial or sporadic childhood deafness referred for molecular diagnostic testing. *Genetics in Medicine*, *4*(4), 279-288.

Yeager, M. (1998). Structure of cardiac gap junction intercellular channels. *Journal of Structural Biology*, *121*(2), 231-245.

TABLES

Table 1. Frequency of the *GJB2* mutated alleles (n= 528 chromosomes).

Variant		Location	Effect	Aleles, No.	Frequency % (n=528)
DNA level	Protein Level				
c.35delG	p.Gly12Valfs*2	N-terminus (frameshift)	Pathogenic	84	15.9
c.101T>C	p.Met34Thr	M1	Controversial	7	1.3
c.380G>A	p.Arg127His	IC	Controversial	6	1.1
c.71G>A	p.Trp24X	M1 (nonsense)	Pathogenic	5	0.9
c.109G>A	p.Val37Ile	M1	Pathogenic	5	0.9
c.139G>T	p.Glu47X	E1 (nonsense)	Pathogenic	3	0.6
c.358_360delGAG	p.Glu120del	IC	Pathogenic	3	0.6
c.516G>A	p.Trp172X	E2 (nonsense)	Pathogenic	3	0.6
c.617A>G	p.Asn206Ser	M4	Pathogenic	3	0.6
c.-23+1G>A	NA	Donor splice site	Pathogenic	2	0.4
c.283G>A	p.Val95Met	M2	Pathogenic	2	0.4
c.-259C>T[a, b]	NA	Basal promoter	Pathogenic	1	0.2
c.-22-12C>T	NA	Intron	Polymorphism	1	0.2
c.24G>A	p.Thr8Thr	N-terminus	Novel	1	0.2
c.167delT	p.Leu56Argfs*26	E1 (frameshift)	Pathogenic	1	0.2
c.250G>A	p.Val84Met	M2	Pathogenic	1	0.2
c.269T>C	p.Leu90Pro	M2	Pathogenic	1	0.2
c.457G>A	p.Val153Ile	M3	Controversial	1	0.2
c.468G>A	p.Gly160Ser	E2	Unclear	1	0.2
c.487A>C[a]	p.Met163Leu	E2	Pathogenic	1	0.2
c.550 C>G	p.Arg184Trp	E2	Pathogenic	1	0.2

a. Mutations identified for the first time in the Portuguese population; b. Mutation up to now private of one Portuguese family.

Table 2. *GJB2* genotypes of the 80 patients carrying *GJB2* mutations.

GJB2 genotype	Probands	Frequency % (n=264)	HL better ear		History
Biallelic					
[c.35delG]+[c.35delG]	28	10,6	5	Moderate	6 FC; 8 SC; 14 Un
			5	Severe	
			11	Profound	
			7	Unknown	
[c.35delG]+[p.Glu47X]	3	1,1	1	Profound	1 FC; 2 Un
			2	Unknown	
[c.35delG]+[p.Trp172X]	3	1,1	1	Moderate	1 FC; 1 SC; 1 Un
			2	Profound	
[c.35delG]+[p.Val37Ile]	2	0,8	1	Mild	1 FC; 1 SC
			1	Moderate	
[c.35delG]+ [c.-23+1G>A]	1	0,4		Profound	Un
[c.35delG]+[p.Trp24X]	1	0,4		Moderate	SC
[c.35delG]+[p.Met34Thr]	1	0,4		Unknown	Un
[c.35delG]+[c.167delT]	1	0,4		Severe	FC
[c.35delG]+[p.Leu90Pro]	1	0,4		Moderate	SC
[c.35delG]+[p.Val95Met]	1	0,4		Profound	SC
[c.35delG]+[delE120]	1	0,4		Moderate	SC
[c.35delG]+[p.Asn206Ser]	1	0,4		Moderate	FC
[p.Trp24X]+[p.Trp24X]	1	0,4		Moderate	FC
[p.Trp24X]+[p.Arg127His]	1	0,4		Moderate	FC
[p.Met34Thr]+[p.Val95Met]	1	0,4		Unknown	Un
[p.Val37Ile]+[p.Arg184Trp]	1	0,4		Severe	SC
[p.Val37Ile]+[p.Asn206Ser]	1	0,4		Severe	SC
[p.Val84Met]+ [c.-259C>T]	1	0,4		Profound	FC
[p.delE120]+[p.delE120]	1	0,4		Moderate	SC
[p.Arg127His]+[c.-23+1G>A]	1	0,4		Profound	SC
[p.Arg127His]+[p.Arg127His]	1	0,4		Unknown	FC
Total	**53**	**20,1**			
Monoallelic					
[=]+[c.35delG]	12	4,5	1	Mild	4 FC; 5 SC; 2 Un
			3	Moderate	
			3	Severe	
			3	Profound	
			2	Unknown	
[=]+[p.Met34Thr]	5	1,9	1	Moderate	4 FC; 1 Un

			1	Severe	
			2	Profound	
			1	Unknown	
[=]+[p.Arg127His]	2	0,8	2	Moderate	1 FC; 1 SC
[=]+[c.-22-12C>T]	1	0,4		Severe	Un
[=]+[p.Thr8Thr]	1	0,4		Unknown	Un
[=]+[p.Trp24X]	1	0,4		Severe	FC
[=]+[p.Val37Ile]	1	0,4		Profound	SC
[=]+[p.Val153Ile]	1	0,4		Mild	FC
[=]+[Gly160Ser]	1	0,4		Moderate	SC
[p.Met163Leu]+[=]	1	0,4		Mild	FC
[=]+[p.Gln206Ser]	1	0,4		Moderate	Un
Total	**27**	**10,2**			

FC=Familiar Case; SC=Sporadic Case; Un=Unknown

CHAPTER 3

A novel mutation (c.-259C>T) impairs GJB2 basal promoter activity

A novel hearing loss-related mutation occurring in the *GJB2* basal promoter

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ABSTRACT

Mutations in the *GJB2* gene are a major cause of nonsyndromic recessive hearing loss in many countries. In a significant fraction of patients, only monoallelic *GJB2* mutations known to be either recessive or of unclear pathogenicity are identified. This paper reports a novel *GJB2* mutation, -3438C→T, found in the basal promoter of the gene, in *trans* with V84M, in a patient with profound hearing impairment. This novel mutation can abolish the basal promoter activity of *GJB2*. These results highlight the importance of extending the mutational screening to regions outside the coding region of *GJB2*.

INTRODUCTION

Hereditary hearing loss is a genetically heterogeneous disorder with 85 loci and 39 nuclear disease genes reported to date.[1] Most of the cases of genetic hearing loss are non-syndromic, and of these, most are autosomal recessive. In some populations, up to 50% of cases of prelingual, non-syndromic sensorineural hearing loss (NSSHL) are due to mutations in a single gene, *GJB2*,[2] which codes for connexin 26 protein (Cx26). Therefore, *GJB2* is normally the first gene to be tested in patients with hearing loss. Mutational screening performed to date has usually focused on the coding region. Few studies have been conducted on the non-coding exon 1 of *GJB2*, and even fewer on the promoter region of this gene. As a result of the *GJB2* screening performed to date, the majority of patients in various populations have been reported with only one *GJB2* mutation, either recessive or of unclear pathogenicity. Some of these cases were elucidated upon screening for the two common *GJB6* deletions.[3–5] The remaining cases possibly have pathogenic mutations yet to be found in the promoter region or other non-coding regions of *GJB2*. Notably, pathogenic mutations have been identified in the 5' untranslated region (UTR) and promoter region of *GJB1* (Cx32),[6–8] another gene of the connexin family. In this paper, we report the genetic identification and functional analysis of the first *GJB2* promoter mutation.

METHODS

Subjects and audiometry

We studied a Portuguese family with NSSHL: the 35-year oldmother and her two daughters (fig 1A). The mother (I-2) and the 12-year proband (II-2), had the hearing evaluated in both ears using pure-tone audiometry. Both presented with bilateral sensorineural hearing loss, but while the mother was only moderately affected, the proband had profound hearing impairment (fig 1B). The other child (II-1) had normal hearing. No biological or audiological data was available for the father (I-1).

Eight unrelated patients, presenting with different degrees of hearing loss, and with only a monoallelic *GJB2* variant previously identified, were also part of this study. Six of them have one controversial *GJB2* variant (M34T, R127H or G160S), and the other two patients harbour one recessive *GBJ2* allele (35delG). None of these eight patients has either of the two *GJB6* deletions, del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854).

The control population used comprised 70 non-related, hearing people from the Portuguese general population.

Informed consent was obtained from each participant before collecting blood for DNA extraction.

Screening of the GJB2 coding region and receptor splice site

The coding region and receptor splice site of *GJB2* gene were analysed by single-strand conformational polymorphism (SSCP) in both affected members of the family (I-2 and II-2). Sequencing of all the *GJB2* coding region and receptor splice site was performed to identify the sequence variation detected by SSCP and to exclude other possible mutations in that genomic region. Before analysis of *GJB2* basal promoter, both I-2 and II-2 were screened for the common *GJB6* deletions, del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854), following the method used by del Castillo *et al*.[4]

Screening of the GJB2 donor splice site, exon 1 and basal promoter

For the three members of the family under study, the 70 Portuguese controls and the eight patients with only a monoallelic *GJB2* variant, a *GJB2* region of 1009 bp, comprising the donor splice site, exon 1 and part of the 5' upstream sequence, was amplified by PCR using the forward primer 5'-CgTTCgTTCggATTggTgAg-3' and the reverse primer 5'-CAgAAACgCCCgCTCCAgAA-3'. The PCR products from the proband and one control patient were sequenced using the internal forward primer 5'-ggCTCAAAggAACTAggAgATCg-3', with the same reverse primer as that used for amplification. These primers, used for sequencing, delimit a 539 bp *GJB2* region (from nucleotides -3573 to -3034 relative to the initiator methionine), which includes the 128 bp basal promoter, exon 1 and the donor splice site.

The presence of the -3438C→T mutation identified in the proband was investigated in her mother and sister, and in the 70 Portuguese controls, by restriction analysis of the PCR products with the enzyme *Bsr*BI (New England Biolabs, Quinta do Paizinho, Carnaxide, Portugal). The eight patients who had only a monoallelic *GJB2* variant were assessed by sequencing for the presence of this mutation or other mutations in the donor splice site, exon 1 or basal promoter. The basal promoter, exon 1 and donor splice site of *GJB2* gene can be found in GenBank (accession number U43932.1).

Reporter-gene assay for assessment of wild-type and mutant GJB2 basal promoter activity

A fragment of the promoter region from *GJB2* alleles containing either a cytosine or a thymine at position -3438 relative to the initiator methionine was amplified by PCR using the forward primer 5'-ATACAgAgCTCACAgAggACAACgACCACAg-3', designed to contain a SacI site, and the reverse primer 5'-TTAgTACCATggAgggCCgCAACACCTgTC-3', designed to contain a *Nco*I site. The PCR products were cloned into the vector pCR2.1-topo using a commercial kit (TOPO TA pCR 2.1, TOPO Cloning Kit (ref. 45-064); Invitrogen, Prat de Llobregat, Barcelona, Spain). The *Sac*I–*Nco*I fragment was excised by double digestion with the enzymes *Sac*I and *Nco*I, and was then directionally cloned into pGL3-basic vector (Promega, Lisbon, Portugal) using its *Sac*I and *Nco*I sites. A fragment upstream of the basal promoter and downstream of the *Sac*I site was

then removed by digestion with *Sac*I and *Xma*I followed by selfligation of the remaining plasmid. The resulting constructs contained, therefore, a fragment of the *GJB2* promoter region ranging from nucleotides -3348 to -3490, which includes the entire basal promoter, cloned upstream of the firefly luciferase coding sequence. The constructs termed -3438C-pGL3 (wildtype sequence) and -3438T-pGL3 (containing the mutation -3438C→T) were assessed by sequencing and a diagnostic digestion with *Bsr*BI to confirm sequence integrity. HEK-293 or Caco-2 cells were transfected with equimolar amounts of pRLTK
vector (Promega) and -3438C-pGL3, -3438T-pGL3 or pGL3-basic, using a commercial transfection reagent (FuGENE 6.0; Roche, Basel, Switzerland). The transfected cells were lysed 48h post-transfection. Luminescence of firefly luciferase (coded by pGL3-basic vector and by the pGL3 constructs) and *Renilla* luciferase (coded by pRL-TK vector) was assessed (Dual-LuciferaseH Reporter Assay System; Promega) by a multilayer counter plate reader (Victor(2) 1420-002; Perkin Elmer Wallack, Norton, Ohio, USA). The reporter gene assay was performed five times on HEK-293 cells and four times on Caco-2 cells. The firefly luciferase luminescence values were normalised against the *Renilla* luciferase luminescence values for each sample in each experiment. For each construct the average value of normalised luciferase luminescence was calculated and plotted in a histogram and analysed using the *t* test and Microsoft Excel software.

Dye-transfer assay for assessment of wtCx26 and p.Val84Met-Cx26 function

Gap-junctional communication in Wt-Cx26 and V84M-Cx26- expressing cells was assessed by intercellular spread of Lucifer yellow (LY) and neurobiotin (NBN). LY and NBN were delivered to HeLa cells using the whole-cell configuration of the patch-clamp technique. HeLa cells were transiently transfected with 500 ng Wt-Cx26-enhanced green fluorescent protein (EGFP) cDNA or V84M-Cx26-EGFP cDNA, using a commercial transfection reagent (Lipofectamine LTX; Invitrogen) according to the manufacturer's recommendations. Transfected HeLa cells grown on 9 mm coverslips were transferred to a recording chamber 24 hours after transfection. Cells in which significant EGFP-positive gap junction plaques were identified were selected for dye transfer. Following 5-minute whole-cell recordings, cells were fixed in 4% paraformaldehyde for

20 minutes and washed in phosphate-buffered saline. LY and NBN were detected and imaged as previously described.[9]

RESULTS

Analysis of the coding region of the *GJB2* gene revealed that the proband and her mother were heterozygous for the V84M mutation (fig 1C). No other coding *GJB2* variants were found. The V84M mutation has been previously reported to be associated with profound hearing loss.[10 11] In the latter study, V84M was in *trans* with the 35delG mutation or M34T, suggesting that the V84M mutation is a recessive *GJB2* mutation associated with profound hearing loss. As the proband analysed in our study was heterozygous for V84M and had profound hearing impairment, we hypothesised that she could yet possess either one of the two common *GJB6* deletions, del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854), or a non-coding *GJB2* mutation. Therefore, we tested the proband and her mother for the presence of del(*GJB6*-D13S1830) or del(*GJB6*-D13S1854). The deletions were not detected in either patient. We then sequenced the *GJB2* donor splice site, exon 1 and basal promoter of the proband. A previously unreported substitution, -3438C→T, was found in the heterozygous state within the basal promoter. Restriction analysis with the enzyme *Bsr*BI revealed that the proband's normal-hearing sister also had one allele -3438C→T, but their mother, who had moderate hearing impairment, did not (data not shown). This variation was not found in the 70 hearing individuals from the Portuguese general population. Neither this nor other mutations were found in the donor splice site, exon 1 or basal promoter of seven of the eight monoallelic patients analysed. However, one monoallelic patient, heterozygous for the controversial R127H variant, was shown to be heterozygous for the donor splice-site recessive mutation IVS1+1G→A.

The novel -3438C→T mutation is localised 3438 nucleotides upstream of the initiator methionine, in the GC box at position -81 relative to the transcription start point (TSP). This GC box and another at position -93 relative to the TSP, regulate *GJB2* basal transcription by interacting with the transcription factors Sp1 and Sp3, but the -81 box is the most important.[12]

Because the -3438C→T mutation disrupts this GC box, we considered it to be potentially pathogenic, and reporter-gene assays were performed to confirm this. They revealed that the -3438C→T mutation abolishes the *GJB2* basal promoter activity in HEK-293 cells (fig 2), and greatly reduces it in Caco-2 cells (fig 3). Figure 2 shows that the mean normalised value of firefly luciferase luminescence from cells transfected with the plasmid containing the wild-type basal promoter (-3438C-pGL3) was higher than and significantly different from the value obtained from cells transfected with the promoterless pGL3-basic vector, which indicates promoter activity of the wild-type basal promoter ($p<0.01$), as expected. In contrast, there was no significant difference of the mean normalised values of firefly luciferase luminescence between cells that were transfected either with the mutant basal promoter (-3438T-pGL3) or with the pGL3-basic vector ($p=0.29$). This means that the mutation -3438C→T abolishes the activity of the *GJB2* basal promoter in HEK-293 cells. In Caco-2 cells, the mutant *GJB2* basal promoter showed some activity compared with the pGL3-basic vector ($p<0.001$), but was much less active than the wild-type basal promoter ($p<<0.001$) (fig 3).

To confirm the suspected pathogenic effect of the V84M mutation we performed a dye-transfer assay in HeLa cells. This experiment revealed clear differences between Wt-Cx26 and V84M-Cx26 function in the permeability of gap junctions to LY and NBN. In both groups of cells, there were detectable gap junction plaques between adjacent cells (fig 4). In all Wt-Cx26 recordings (6/6) there was LY and NBN transfer to at least one adjacent cell (fig 4A). Conversely, in all V84M-Cx26 recordings (8/8) there was no transfer of LY or NBN (fig 4B). The membrane capacitance (an approximation of the total continuous membrane area in contact with the recording pipette) was significantly larger in Wt-Cx26 recordings (mean (SD) 54.8 (8.7) pF, n=6) compared with V84M-Cx26 recordings (29.6 (2.9) pF, n=8, $p<0.01$; unpaired *t* test). These results suggest that cells expressing V84M-Cx26 can form gap junction-like aggregates at the plasma membrane, but this does not allow intercellular electrical or molecular coupling.

DISCUSSION

Connexin 26, a transmembrane protein coded by the gene *GJB2*, and a component of gap junctions, is strongly expressed in the cochlea, both in epithelial and connective tissues, where it is believed not only to have a role in the recirculation of the ion K^+,[13] which is a crucial mechanism for the transduction of sound waves into nervous impulses and consequently for proper hearing function, but also in the permeability to signalling molecules and metabolites.[14] The gene *GJB2* is composed of two exons separated by an intron, and the coding region is entirely contained in exon 2. The basal promoter activity resides in the first 128 nucleotides upstream of the TSP and has two GC boxes, at positions -81 and -93 from the TSP, which are important for transcription.[12]

Most of the *GJB2* sequence variations described to date are localised in the coding region, and only a few have been reported in non-coding regions of the gene.[15–19] Among the latter, there are two donor splice-site recessive mutations.[15 19] The rest of the non-coding variations reported to date are localised upstream of the exon 1, in exon 1, the intron, the 5' UTR of exon 2, or the 3' UTR, but none of these have yet been proven to be pathogenic. One is the -493del10 deletion,[16] located upstream of the basal promoter region, which occurs in most of the *GJB2* alleles harbouring the M34T mutation. This deletion disrupts a MGF-like sequence that is strongly homologous to the MGF binding site of the mouse β-casein gene.[20] It was found that *GJB2* alleles containing both -493del10 and M34T were expressed in cultured keratinocytes,[16] but it is not known whether the deletion altered the gene's normal expression and whether it has an effect in the cochlea.

We found a novel sequence variation in the gene *GJB2* within the basal promoter, more specifically, in the -81 GC box. This novel mutation, -3438 C→T, was found in *trans* with the V84M mutation in a patient with profound hearing impairment, the proband of this study. Her mother, who had moderate hearing impairment, harboured V84M as the sole mutation in the coding region of *GJB2*, and tested negative for the -3438C→T mutation. The proband's normal-hearing sister did not have the V84M, but was found to be heterozygous for the -3438C→T mutation. The V84M mutation has been reported in other cases of profound hearing loss,[10 11]

and one of these cases was a patient also harbouring the 35delG,[11] which suggests that when V84M-Cx26 is the only variant of Cx26 being produced, profound hearing loss can result. As the -81 GC box was found to be crucial for the transcription of the gene in both the MCF-12A (human immortalised mammary epithelial) and RL95-2 (human endometrial) cell lines,[12] we hypothesised that the -3438 C→T mutation could cause loss of function of the GC box, impairing or abolishing the *GJB2* transcription in the cochlea. In this way, the profound hearing loss of the proband analysed here could be due to expression of only the V84MCx26 protein. To confirm that the *GJB2* genotype -3438C→T/V84M could indeed be the cause of the profound hearing loss of the proband analysed in this study, we performed functional studies on both mutations.

The reporter-gene assay, performed to assess the functionality of the basal promoter containing the -3438C→T mutation, revealed that this mutation abolishes the *GJB2* basal promoter activity in the HEK-293 cell line and greatly reduces it in the Caco-2 cell line.

The dye-transfer assay of V84M-Cx26 gap-junction properties revealed that this mutation results in communicationincompetent cells. A recent study has suggested that homomeric expression of V84L-Cx26 results in gap-junction channels that have unitary channel conductance and LY permeability that is indistinguishable from that of Wt-Cx26 channels.[14] However, the basis of the hearing impairment caused by thismutation has been ascribed to impaired permeability to inositol trisphosphate. This would result in dysfunctional Ca^{2+} mobilisation in affected cochlear epithelial cells. In our study, HeLa cells expressing V84M-Cx26 also formed morphologically normal gap-junction plaques, but appeared to be uncoupled both electrically (based on membrane capacitance measurements), and metabolically (based on impaired LY and NBN transfer).

In conclusion, this study describes the first pathogenic, hearing loss-related mutation impairing the basal promoter activity of the gene *GJB2*. It also shows that this or other basal promoter mutations, or even mutations in other non-coding regions, may be present in some patients with hearing loss with only a monoallelic *GJB2* recessive mutation. Thus, screening of the basal promoter and other non-coding regions of the *GJB2* gene, such as exon 1, UTRs and both splice sites, are important and necessary for improving genetic diagnosis regarding these

monoallelic patients, and for subsequent genetic counselling. We also present the first functional evidence for the pathogenicity of the previously described V84M mutation.

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REFERENCES

1 Petersen MB, Willems PJ. Non-syndromic, autosomal-recessive deafness. *Clin Genet* 2006;**69**:371–92.

2 Kenneson A, Van Naarden Braun K, Boyle C. GJB2 (connexin 26) variants and nonsyndromic sensorineural hearing loss: a HuGE review. *Genet Med* 2002;**4**:258–74.

3 del Castillo I, Villamar M, Moreno-Pelayo MA, del Castillo FJ, Alvarez A, Telleria D, Menendez I, Moreno F. A deletion involving the connexin 30 gene in nonsyndromic hearing impairment. *N Engl J Med* 2002;**346**:243–9.

4 del Castillo FJ, Rodriguez-Ballesteros M, Alvarez A, Hutchin T, Leonardi E, de Oliveira CA, Azaiez H, Brownstein Z, Avenarius MR, Marlin S, Pandya A, Shahin H, Siemering KR, Weil D, Wuyts W, Aguirre LA, Martin Y, Moreno-Pelayo MA, Villamar M, Avraham KB, Dahl HH, Kanaan M, Nance WE, Petit C, Smith RJ, Van Camp G, Sartorato EL, Murgia A, Moreno F, del Castillo I. A novel deletion involving the connexin-30 gene, del(GJB6-d13s1854), found in trans with mutations in the GJB2 gene (connexin-26) in subjects with DFNB1 nonsyndromic hearing impairment. *J Med Genet* 2005;**42**:588–94.

5 del Castillo I, Moreno-Pelayo MA, Del Castillo FJ, Brownstein Z, Marlin S, Adina Q, Cockburn DJ, Pandya A, Siemering KR, Chamberlin GP, Ballana E, Wuyts W, Maciel-Guerra AT, Alvarez A, Villamar M, Shohat M, Abeliovich D, Dahl HH, Estivill X, Gasparini P, Hutchin T, Nance WE, Sartorato EL, Smith RJ, Van Camp G, Avraham KB, Petit C, Moreno F. Prevalence and evolutionary origins of the del(GJB6-D13S1830) mutation in the DFNB1 locus in hearing-impaired subjects: a multicenter study. *Am J Hum Genet* 2003;**73**:1452–8.

6 Houlden H, Girard M, Cockerell C, Ingram D, Wood NW, Goossens M, Walker RW, Reilly MM. Connexin 32 promoter P2 mutations: a mechanism of peripheral nerve dysfunction. *Ann Neurol* 2004;**56**:730–4.

7 IonasescuVV, Searby C, Ionasescu R, Neuhaus IM, Werner R. Mutations of the noncoding region of the connexin32 gene in X-linked dominant Charcot-Marie-Tooth neuropathy. *Neurology* 1996;**47**:541–4.

8 Wang HL, Wu T, Chang WT, Li AH, Chen MS, Wu CY, Fang W. Point mutation associated with X-linked dominant Charcot-Marie-Tooth disease impairs the P2 promoter activity of human connexin-32 gene. *Brain Res Mol Brain Res* 2000;**78**:146–53.

9 Jagger DJ, Forge A. Compartmentalized and signal-selective gap junctional coupling in the hearing cochlea. *J Neurosci* 2006;**26**:1260–8.

10 Pandya A, Arnos KS, Xia XJ, Welch KO, Blanton SH, Friedman TB, Garcia Sanchez G, Liu MX, Morell R, Nance WE. Frequency and distribution of GJB2 (connexin 26) and GJB6 (connexin 30) mutations in a large North American repository of deaf probands. *Genet Med* 2003;**5**:295–303.

11 Cheng X, Li L, Brashears S, Morlet T, Ng SS, Berlin C, Hood L, Keats B. Connexin26 variants and auditory neuropathy/dys-synchrony among children in schools for the deaf. *Am J Med Genet A* 2005;**139**:13–18.

12 Tu ZJ, Kiang DT. Mapping and characterization of the basal promoter of the human connexin26 gene. *Biochim Biophys Acta* 1998;**1443**:169–81.

13 Kikuchi T, Kimar RS, Paul DL, Adams JC. Gap junctions in the rat cochlea: immunohistochemical and ultrastructural analysis. *Anat Embryol* 1995;**191**:101–18.

14 Beltramello M, Piazza V, Bukauskas FF, Pozzan T, Mammano F. Impaired permeability to Ins(1,4,5)P3 in a mutant connexin underlies recessive hereditary deafness. *Nat Cell Biol* 2005;**7**:63–9.

15 Denoyelle F, Marlin S, Weil D, Moatti L, Chauvin P, Garabedian EN, Petit C. Clinical features of the prevalent form of childhood deafness, DFNB1, due to a connexin-26 gene defect: implications for genetic counselling. *Lancet* 1999;**353**:1298–303.

16 Houseman MJ, Ellis LA, Pagnamenta A, Di WL, Rickard S, Osborn AH, Dahl HH, Taylor GR, Bitner-Glindzicz M, Reardon W, Mueller RF, Kelsell DP. Genetic analysis of the connexin-26 M34T variant: identification of genotype M34T/M34T segregating with mild-moderate non-syndromic sensorineural hearing loss. *J Med Genet* 2001;**38**:20–5.

17 Roux AF, Pallares-Ruiz N, Vielle A, Faugere V, Templin C, Leprevost D, Artieres F, Lina G, Molinari N, Blanchet P, Mondain M, Claustres M. Molecular epidemiology of DFNB1 deafness in France. *BMC Med Genet* 2004;**5**:5.

18 Tang HY, Fang P, Ward PA, Schmitt E, Darilek S, Manolidis S, Oghalai JS, Roa BB, Alford RL. DNA sequence analysis of GJB2, encoding connexin 26: observations from a population of hearing impaired cases and variable carrier rates, complex genotypes, and ethnic stratification of alleles among controls. *Am J Med Genet A* 2006;**140**:2401–15.

19 Green GE, Scott DA, McDonald JM, Woodworth GG, Sheffield VC, Smith RJ. Carrier rates in the midwestern United States for GJB2 mutations causing inherited deafness. *JAMA* 1999;**281**:2211–16.

20 Kiang DT, Jin N, Tu ZJ, Lin HH. Upstream genomic sequence of the human connexin26 gene. *Gene* 1997;**199**:165–71.

FIGURES

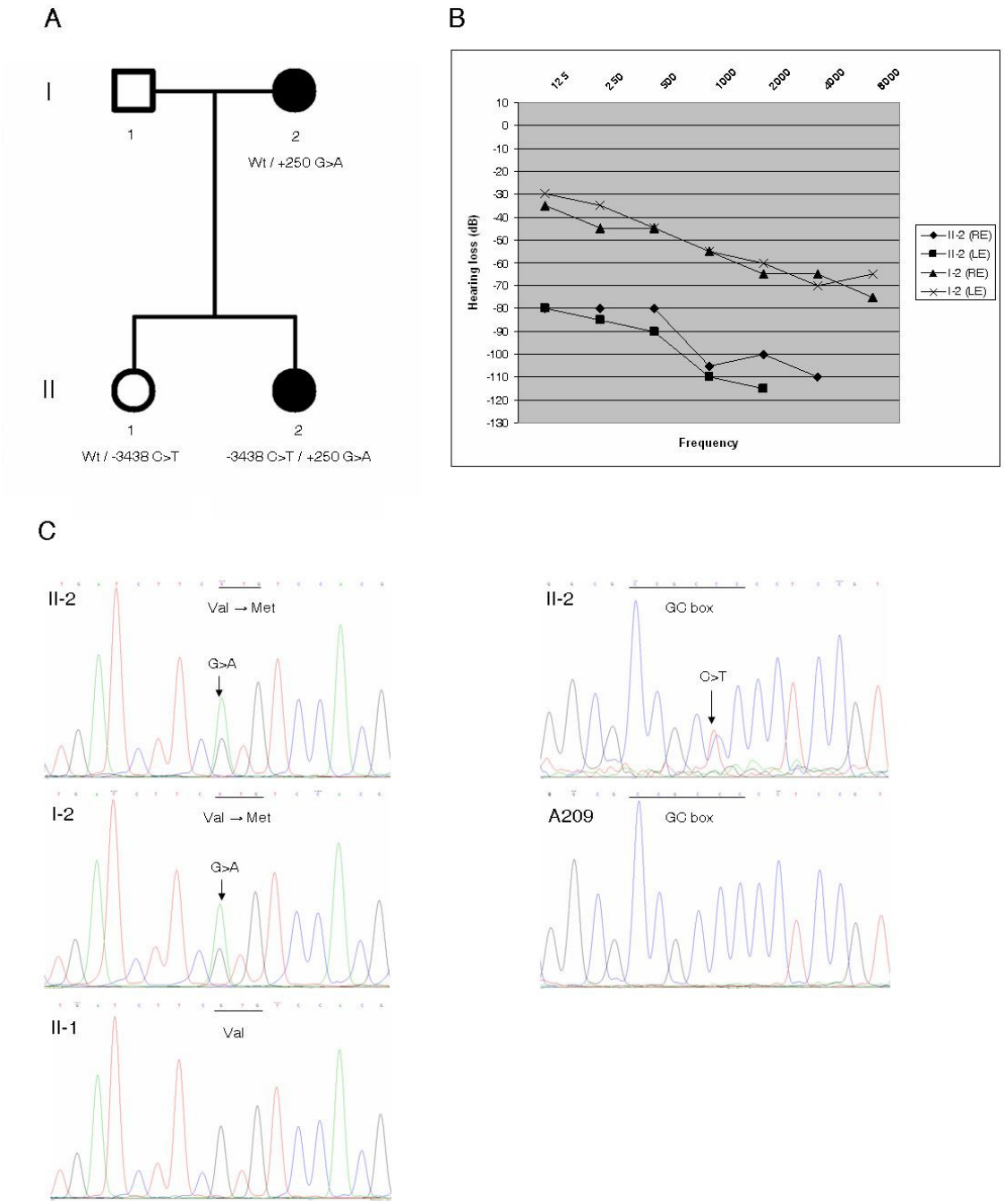


Figure 1. (A) Pedigree of the family here analysed. Black symbols, affected family members; *GJB2* genotypes are displayed below the patients' symbols. (B) Audiograms from right ear (RE) and left ear (LE)

of the affected members (II-2 and I-2), at the ages of 12 and 35 years, respectively. Patient II-2 has bilateral profound hearing loss, and patient I-2 has bilateral moderate hearing impairment. The hearing ability of both patients decreases across the frequencies tested, being most compromised at the high frequencies. (C) Electrophoreograms showing a heterozygous nucleotide change, +250 G→A, resulting in the V84M mutation, in I-2 and II-2; this substitution is not present in II-1, Patient II-2 is also heterozygous for the -3438C→T mutation in the basal promoter region, changing the GC box sequence from CCGCCC to CCGCTC. This alteration is not present in the control individual, A209.

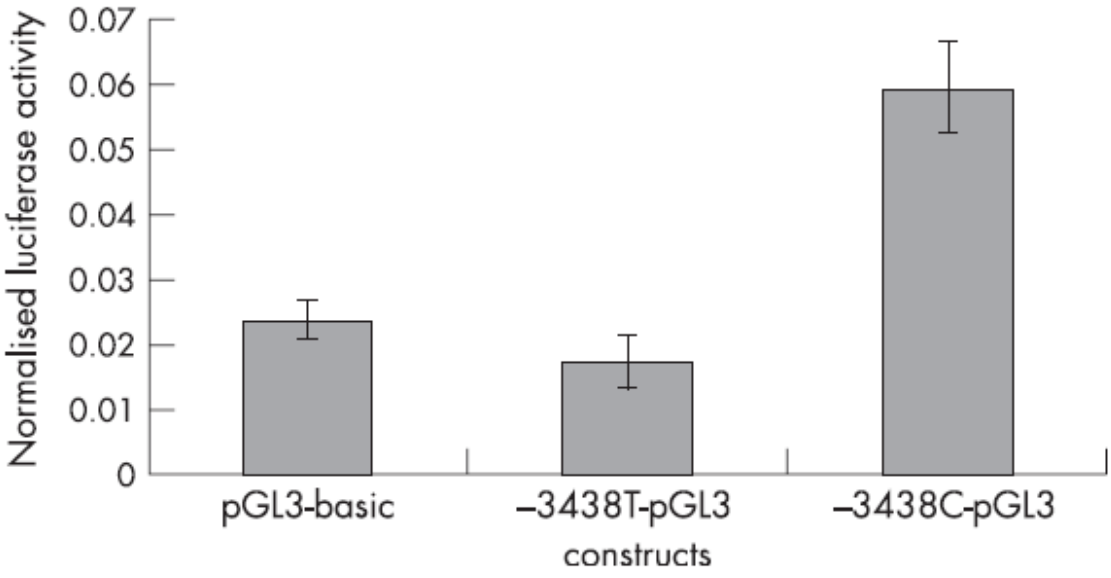


Figure 2. Reporter-gene assay in HEK-293 cells.

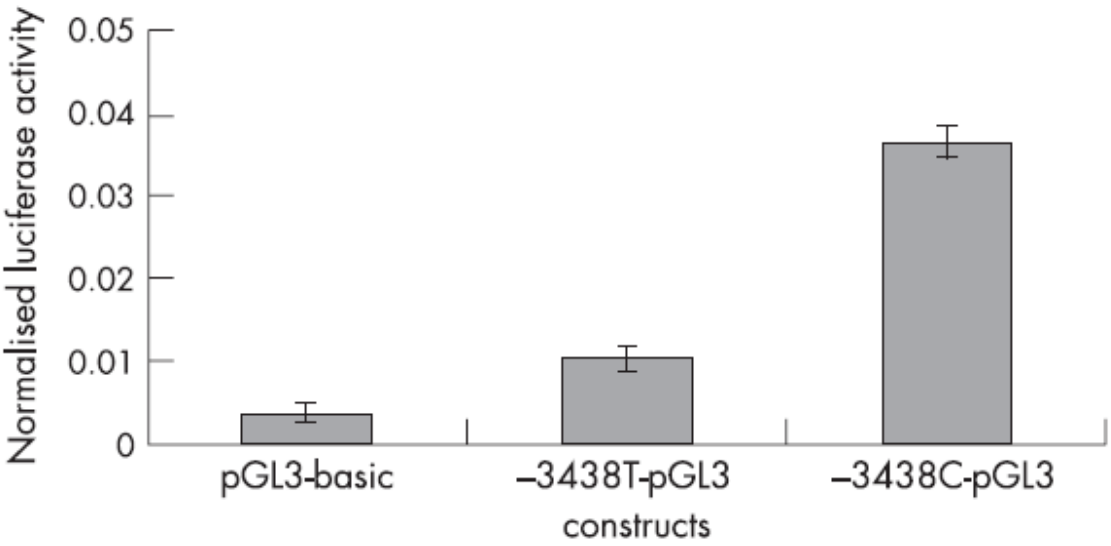


Figure 3. Reporter-gene assay in Caco-2 cells.

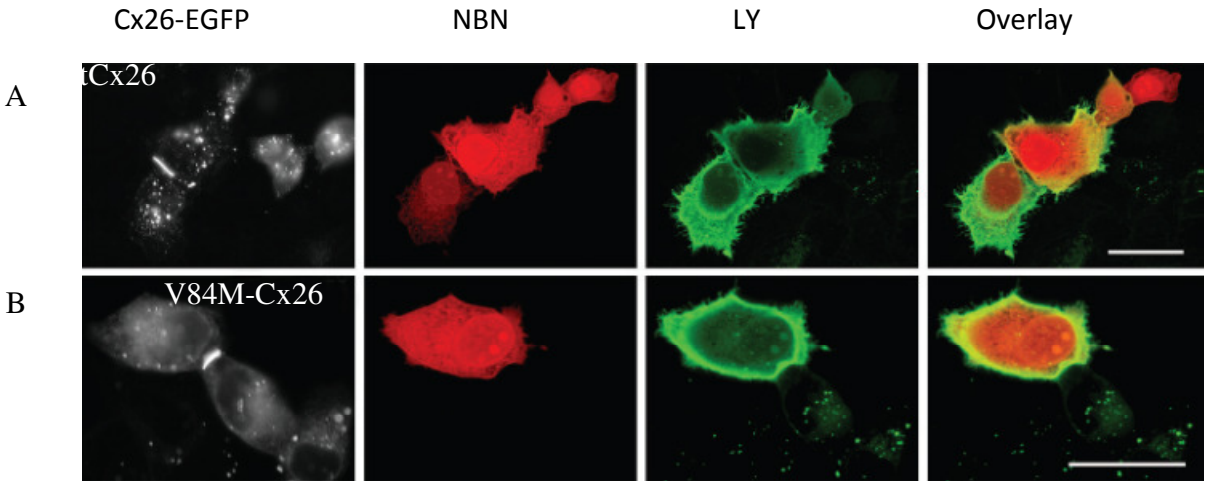


Figure 4. Dye-transfer assay of connexin (Cx) 26 function in Hela cells. (A) In cells expressing Wt-Cx26, enhanced green fluorescent protein (EGFP)-labelled gap junction plaques (arrow) were seen. Following whole-cell dye injections, neurobiotin and Lucifer yellow were seen to transfer to adjacent cells. (B) In cells expressing V84M-Cx26, EGFP-labelled gap junction plaques (arrow) were also apparent. There was no spread of neurobiotin or Lucifer yellow in these cells. Scale bars, 20 µm.

SUPPLEMENTARY INFORMATION

Materials and Methods

Cell culture

Three cell lines were used: HeLa, HEK-293 and NEB1. The first two cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and L-glutamine; they were incubated in 10% CO2, at 37°C. NEB1 cells were cultured in DMEM/F12 (1:1) medium supplemented with 10% FBS, and L-glutamine; they were incubated in 5% CO2, at 37°C.

ANNEX

Figures

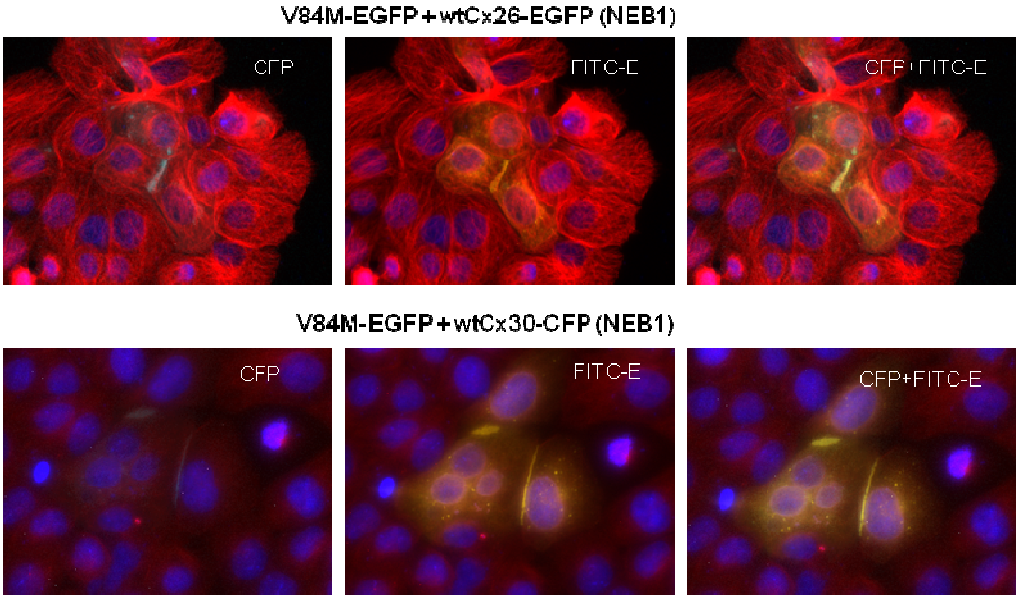


Figure 1. Microscopy images of the co-localisation of p.Val84Met-Cx26 with either wtCx26 or wtCx30 at gap junction-like structures in NEB1 cells.

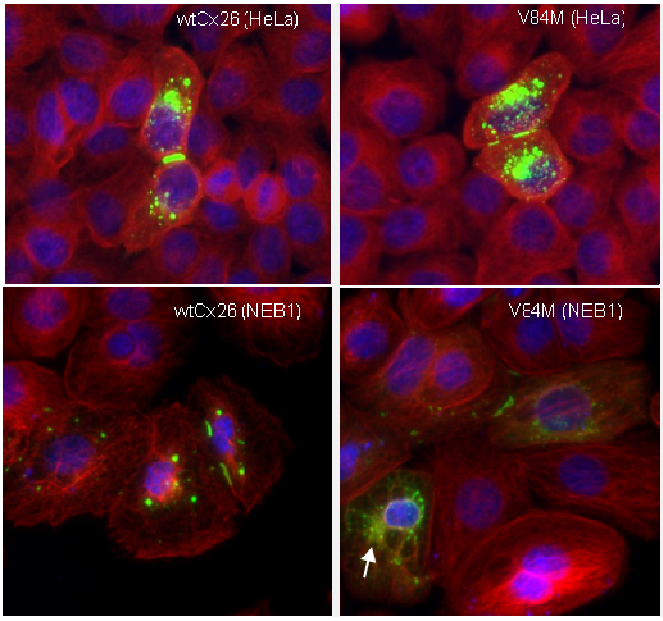


Figure 2. Microscopy images showing the cellular localisation of EGFP-tagged wtCx26 and p.Val84Met-Cx26 in HeLa and NEB1 cells. wtCx26 and p.Val84Met-Cx26 both traffic to cell membrane, forming gap junction-like structures, although the latter protein sometimes appears to be partially retained in the cytoplasm (arrow). Alpha-tubulin is seen in red, and the nucleus is seen in blue.

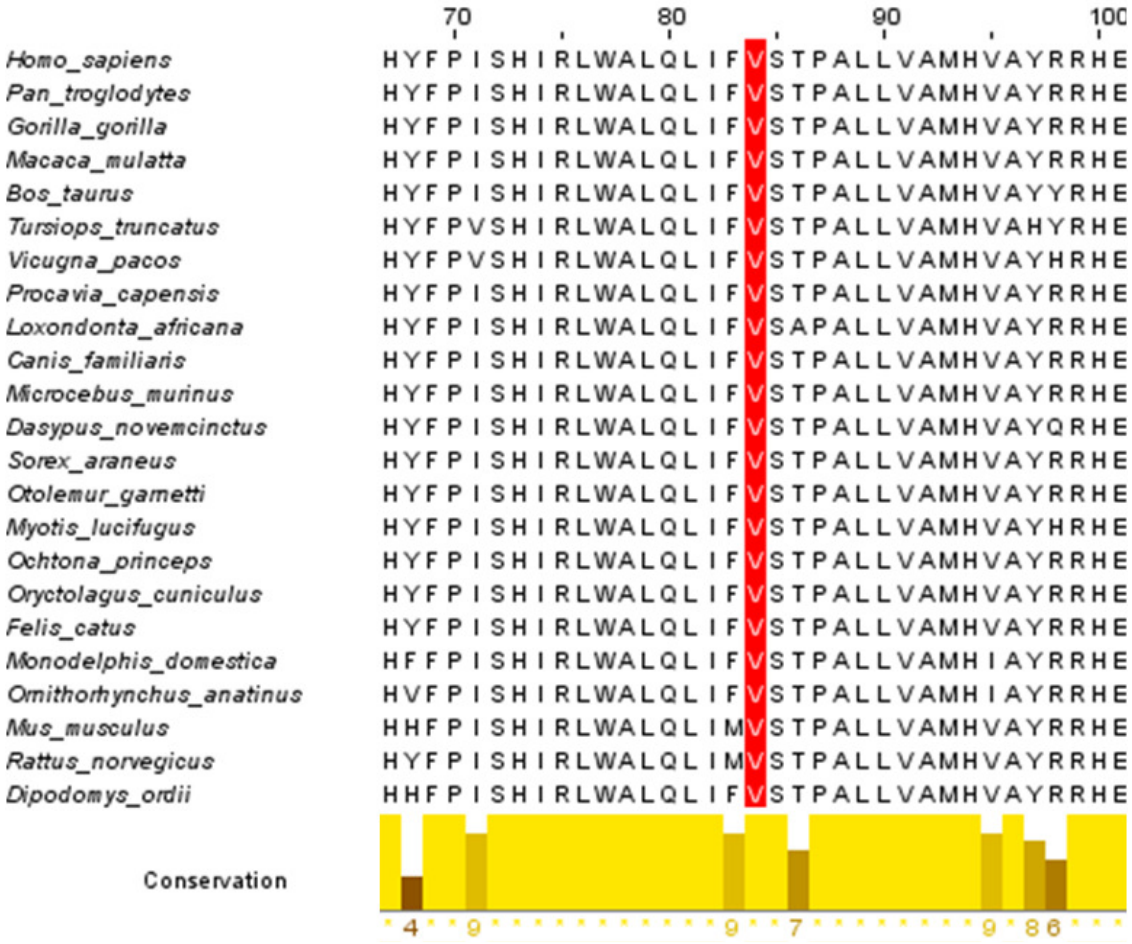
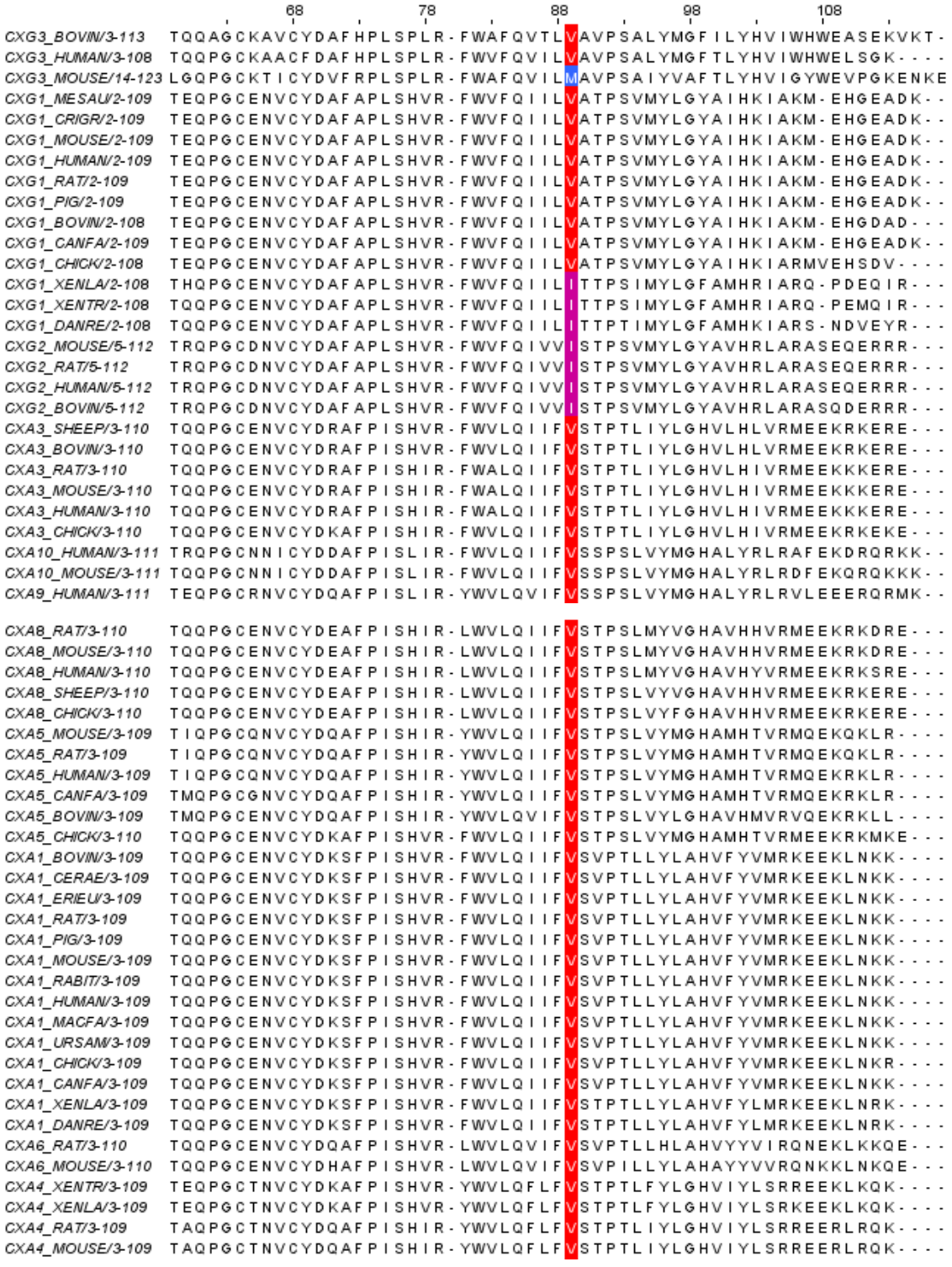


Figure 3. Multiple alignment by ClustalW 2.1(Larkin *et al*., 2007) of Cx26 sequences from 23 mammalian species, visualized in JalView 2.6.1(Waterhouse, Procter, Martin, Clamp, & Barton, 2009). The valine at position 84 (highlighted in red) of Cx26 is conserved. The sequences were retrieved from Ensembl database (Flicek *et al*., 2011).


68
78
88
98
108
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CXG3_MOUSE/14-123 LGQPGCKTICYDVFRPLSPLR-FWAFQVILMAVPSAIYVAFTLYHVIGYWEVPGKENKE
CXG1_MESAU/2-109 TEQPGCENVCYDAFAPLSHVR-FWVFQIILVATPSVMYLGYAIHKIAKM-EHGEADK--
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CXG2_RAT/5-112 TRQPGCDNVCYDAFAPLSHVR-FWVFQIVVISTPSVMYLGYAVHRLARASEQERRR---
CXG2_HUMAN/5-112 TRQPGCDNVCYDAFAPLSHVR-FWVFQIVVISTPSVMYLGYAVHRLARASEQERRR---
CXG2_BOVIN/5-112 TRQPGCDNVCYDAFAPLSHVR-FWVFQIVVISTPSVMYLGYAVHRLARASQDERRR---
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CXA1_MACFA/3-109 TQQPGCENVCYDKSFPISHVR-FWVLQIIFVSVPTLLYLAHVFYVMRKEEKLNKK----
CXA1_URSAM/3-109 TQQPGCENVCYDKSFPISHVR-FWVLQIIFVSVPTLLYLAHVFYVMRKEEKLNKK----
CXA1_CHICK/3-109 TQQPGCENVCYDKSFPISHVR-FWVLQIIFVSVPTLLYLAHVFYVMRKEEKLNKR----
CXA1_CANFA/3-109 TQQPGCENVCYDKSFPISHVR-FWVLQIIFVSVPTLLYLAHVFYVMRKEEKLNKK----
CXA1_XENLA/3-109 TQQPGCENVCYDKSFPISHVR-FWVLQIIFVSTPTLLYLAHVFYLMRKEEKLNRK----
CXA1_DANRE/3-109 TQQPGCENVCYDKSFPISHVR-FWVLQIIFVSTPTLLYLAHVFYLMRKEEKLNRK----
CXA6_RAT/3-110 TQQPGCENVCYDQAFPISHVR-LWVLQVIFVSVPTLLHLAHVYYVIRQNEKLKKQE---
CXA6_MOUSE/3-110 TQQPGCENVCYDHAFPISHVR-LWVLQVIFVSVPILLYLAHAYYVVRQNKKLNKQE---
CXA4_XENTR/3-109 TEQPGCTNVCYDKAFPISHVR-YWVLQFLFVSTPTLFYLGHVIYLSRREEKLKQK----
CXA4_XENLA/3-109 TEQPGCTNVCYDKAFPISHVR-YWVLQFLFVSTPTLFYLGHVIYLSRKEEKLKQK----
CXA4_RAT/3-109 TAQPGCTNVCYDQAFPISHIR-YWVLQFLFVSTPTLIYLGHVIYLSRREERLRQK----
CXA4_MOUSE/3-109 TAQPGCTNVCYDQAFPISHIR-YWVLQFLFVSTPTLIYLGHVIYLSRREERLRQK----

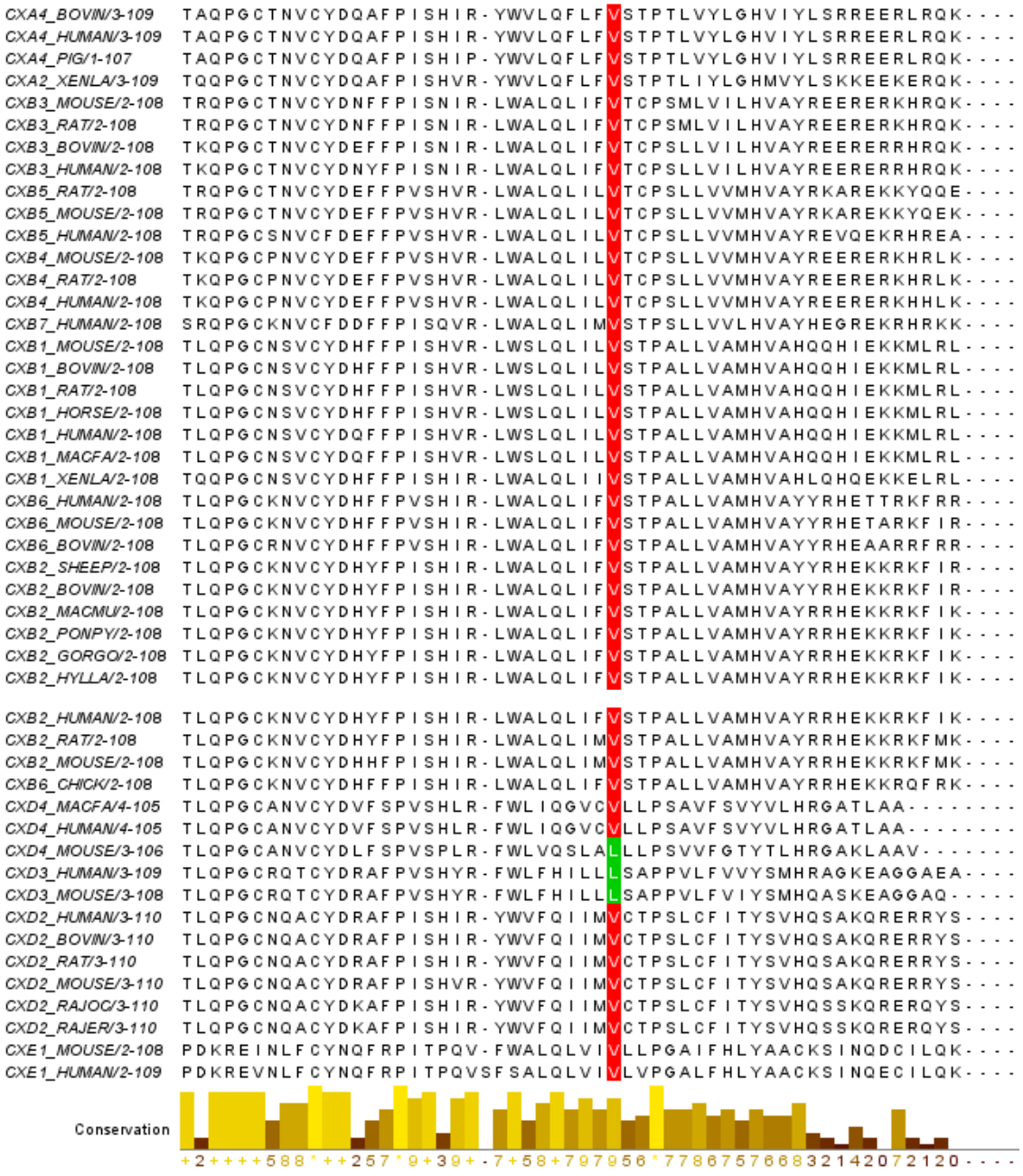

Figure 4. Multiple alignment of connexin sequences in ClustalW 2.1 (Larkin *et al.*, 2007), visualized in JalView 2.6.1 (Waterhouse *et al.*, 2009). The sequences are a subset of the full core composed of 424 connexin partial sequences, containing a connexin signature, therefore grouped as the PF00029 family, in Pfam database (Finn *et al.*, 2010). This subset consists in those sequences that included the position equivalent to the Val84 of Cx26 (highlighted residue column), and to which a classification as to the type

of connexin (alpha, beta, delta, epsilon or gamma: CXA, CXB, CXD, CXE, CXG, respectively) was assigned. The accession name of each sequence (and its length) is provided.

References

Finn, R. D., Mistry, J., Tate, J., Coggill, P., Heger, A., Pollington, J. E., Gavin, O. L., *et al*. (2010). The Pfam protein families database. *Nucleic Acids Research*, *38*(Database issue), D211-D222.

Flicek, P., Amode, M. R., Barrell, D., Beal, K., Brent, S., Chen, Y., Clapham, P., *et al*. (2011). Ensembl 2011. *Nucleic Acids Research*, *39*(Database issue), D800-D806.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., *et al*. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, *23*(21), 2947-2948.

Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M., & Barton, G. J. (2009). Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics*, *25*(9), 1189-1191.

CHAPTER 4

A dominant GJB2 mutation (p.Met163Leu) causes cell death

A novel M163L mutation in connexin 26 causing cell death and associated with autosomal dominant hearing loss

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ABSTRACT

Mutations in *GJB2* gene (encoding connexin 26) are the most common cause of hereditary non-syndromic sensorineural hearing loss (NSSHL) in different populations. The majority of *GJB2* mutations are recessive, but a few dominant mutations have been associated with hearing loss either isolated or associated with skin disease. We describe a novel dominant pathogenic *GJB2* mutation, identified in a Portuguese family affected with bilateral mild/moderate high-frequency NSSHL. *In vitro* functional studies demonstrate that the mutant protein (p.M163L) has defective trafficking to the plasma membrane and is associated with increased cell death.

1. INTRODUCTION

The *GJB2* gene encodes for connexin 26 (Cx26) which belongs to the connexin protein family. This protein oligomerizes in groups of six subunits making up the connexons. Each connexon docks into another one from an apposing cell, forming an intercellular channel. These channels cluster into structures named gap junctions, which allow metabolites and messenger molecules to pass between cells for synchronised function and ionic homeostasis of tissues and organs.

In the cochlea, Cx26 is expressed in the supporting cells of the organ of Corti, the fibrocytes of the spiral ligament and the basal cells of *stria vascularis* (Forge *et al*., 2003) and may play a role in K^+ recycling (Wangemann, 2002). This cyclic ionic flux is crucial for the auditory process since it enables the transduction of sound into nerve impulses which are then directed to the brain for processing.

A broad spectrum of *GJB2* mutations associated with hearing loss have been reported (Cooper *et al*., 2007), accounting for up to 50% of the cases of NSSHL occurring in many populations. Most of the *GJB2* mutations described so far are recessive but a few dominant variants associated to both syndromic and non-syndromic hearing impairment have also been identified (Ballana *et al*., 2008).The identification of additional dominant *GJB2* mutations might

help in understanding the relationship between this gene and disease phenotypes. Here we report a novel apparently dominant *GJB2* mutation identified in a Portuguese nuclear family with bilateral NSSHL. Functional aspects of the corresponding Cx26 protein variant (p.M163L) have been investigated.

2. MATERIALS AND METHODS

A nuclear Portuguese family with a possible dominant form of NSSHL (Fig. 1) was studied. The three family members underwent otoscopic examination and audiometric testing. Hearing levels were determined by pure-tone audiometry with a diagnostic audiometer in a soundproof room. Environmental causes (for example, infectious diseases and ototoxic drugs) were excluded by interview. Individual I-2 had unilateral hearing loss associated with chronic otitis. The affected individuals I-1 and II-1 presented with similar audiological phenotypes (Fig. 1).

2.1. Mutation screening

The members of this Portuguese family were first tested for the 35delG mutation, and then screened for mutations in the whole *GJB2* coding region and flanking sequences, including the acceptor splice site, by Single-Strand Conformational Polymorphism (SSCP), according to Scott *et al*. (1998). Following detection of sequence variations by SSCP, direct sequencing of the *GJB2* coding region and acceptor splice site was performed. The novel M163L mutation was confirmed by PCR, using the primers Cx26-17(F) and Cx26-5(R) (Scott *et al*., 1998), followed by enzymatic restriction with the enzyme *Pst*I (New England Biolabs) in a 20 µL reaction volume at 37°C for 3 h. The same enzymatic restriction analysis was carried out in 103 non-related normal hearing control individuals from the Portuguese population. Mutational screening of *GJB2* basal promoter, exon 1 and donor splice site was performed according to Matos *et al*. (2007).

The *GJB6* deletions del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) and the A1555G and A7445G hearing loss related mtDNA mutations were assessed as described by Del Castillo *et al*. (2005), Estivill *et al*. (1998) and Fischel-Ghodsian *et al*. (1995), respectively.

2.2. Residue conservation analysis

The evolutionary conservation of the Cx26 methionine residue at position 163 was assessed by performing a multiple sequence alignment of the Cx26 sequences from 23 mammalian species, which were retrieved from either Ensembl (http://www.ensembl.org) or UniProt (http://www.uniprot.org) databases. The software ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was used for generating the multiple sequence alignment, and Jalview (Clamp *et al*., 2004) was used for its visualization.

2.3. Functional studies

The behaviour and effects of the mutant M163L-Cx26 protein upon exogeneous expression in HeLa cells, HEK-293 cells (immortalized human embryonic kidney cells) and NEB1 cells (immortalized normal keratinocytes) were studied using microscopy and Fluorescence Activated Cell Sorting (FACS) techniques. All transfection experiments were performed using the Fugene 6 reagent (Roche).

The c.487A>C mutation (p.M163L) was created by Site Directed Mutagenesis, using a QuickChange® Site-Directed Mutagenesis Kit (Stratagene), in the wild-type coding sequence of the *GJB2* gene, previously cloned by Common *et al*. (2004) into the pEGFP-N3 (Clontech) expression vector, in C-terminal fusion with eGFP. The sequence of the resulting vector was confirmed by enzymatic restriction and by sequencing. The construct encoding wild-type Cx26 and constructs encoding eGFP, eGFP-tagged V84M-Cx26, eGFP-tagged D50N-Cx26 and eCFP-tagged wtCx26 or wtCx30, were also used. V84M is a Cx26 non-syndromic hearing loss mutation (Matos *et al*., 2007) and D50N is a Cx26 syndromic skin disease-associated mutation that induces cell death *in vitro* (Common *et al*., 2004).

The cellular localization of the M163L mutant protein was investigated and compared with that of wtCx26 by microscopy following transfection into HeLa and NEB1 cells. Co-localization studies with wtCx26 or wtCx30 and M163L-Cx26 were also performed in NEB1 cells. Cells used for cellular localization experiments were cultured at physiological extracellular calcium concentrations (1.8 mM for HeLa cells and 1.05 mM for NEB1 cells). For assessment of cell death, cells were first transfected with the constructs and maintained either at a physiological

or a high extracellular calcium concentration, prior to being analysed by FACS. Cell sorting was based on the fluorescence of eGFP, and also on the fluorescence of propidium iodide (PI) which was administrated to the cells 2 minutes before being analysed in the FACS machine. Since PI is uptaken by dead cells only, it was possible to sort and quantify the cells into four groups based on eGFP and PI fluorescence (positive or negative), and therefore calculate the percentage of eGFP positive cells which were dead.

The death rates of HEK-293 cells transiently expressing eGFP-tagged M163L-Cx26, V84M-Cx26, D50N-Cx26, or wtCx26, maintained at either a physiological (1.8 mM) or a high (3.6 mM) extracellular calcium concentration, were first determined. In addition, the death rates of NEB1 cells transfected with the same constructs, and maintained at an extracellular calcium concentration within the physiological range (1.05 mM), were also assessed. Vectors encoding eGFP only, eGFP-tagged V84M-Cx26 and D50N-Cx26 were used as controls in these two experiments. Another cell death determination assay was performed using HEK-293 cells transfected with eGFP-tagged M163L-Cx26, wtCx26 and wtCx30, maintained at a physiological (1.8 mM) extracellular calcium concentration. In this latter assay, the total DNA transfected each time was 1 µg; when two constructs were co-transfected the ratio used was 1:1, and when cells were co-transfected with M163L-Cx26, wtCx26 and wtCx30 the ratio used was 1:1:2, respectively. NEB1 cells were cultured at a distinct physiological extracellular calcium concentration (1.05 mM) since this is the calcium concentration present in the standard media used to grow this cell line.

3. RESULTS

3.1. Audiological findings

The audiogram shapes of mother (I-1) and daughter (II-1) are quite similar, showing a ‘‘ski-slope” hearing loss (Fig. 1). Both present with high-frequency hearing loss, which is bilaterally moderate in the mother, and is mild in the left ear/moderate in the right ear in the daughter. The father (I-2) has unilateral profound hearing loss linked with chronic otitis.

3.2. Mutational screening

The father (I-2) was heterozygous for c.35delG. However, a novel *GJB2* mutation, the transversion c.487A > C, was identified in heterozygosity in the mother (I-1) and daughter (II-1), but not in the father (I-2). This mutation caused the substitution of the methionine amino acid residue at position 163 of the Cx26 protein for a leucine (p.M163L) has not previously been reported in the literature and was not found in the normal hearing control samples tested (0/206 control alleles). DNA from the mother and daughter was also screened for mutations in the basal promoter, exon 1 and donor splice site of the *GJB2* gene, as well as for the deletions del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) in the *GJB6* gene. As maternal inheritance could not be excluded, the presence of A1555G and A7445G mutations in the mtDNA was assessed. No further mutations were detected in these patients.

3.3. Residue conservation analysis

The Cx26 sequences from 23 mammalian species were aligned using ClustalW, in order to investigate the evolutionary conservation of the methionine residue, changed by the M163L mutation, located at position 163 of the protein. The multiple alignment revealed a total conservation of this residue across all the species, while some of its nearest residues show interspecific variation.

3.4. Functional studies

The M163L mutant protein was studied by microscopy, in regard to its trafficking and cellular localization, in HeLa and NEB1 cells. Contrary to wtCx26-EGFP, nearly no trafficking to the plasma membrane was observed with the M163L mutant having a predominantly punctate pattern in the cytoplasm (Fig. 2). However, when co-expressed with wtCx26-CFP or wtCx30-CFP, the mutant connexin was able to traffic to cell membrane, co-localizing with the CFP-tagged wild type connexins (Fig. 3) similar to other non-trafficking Cx26 mutations previously studied (Marziano *et al*., 2003; Di *et al*., 2005).

Microscopic observation also revealed that cells expressing M163L-Cx26 alone acquired a rounded and detached morphology which suggested the cells were dying (Fig. 2). This

microscopic observation and a report on another hearing loss associated Cx26 mutation, G45E, which forms abnormally open hemi-channels and thus causes cell death at physiological but not at high extracellular calcium concentrations (Stong *et al*., 2006), raised the possibility that M163L mutation induces the same kind of hemichannel anomaly. Therefore, FACS analysis was used to investigate the effect of M163L-Cx26 on cell viability, under physiological and high extracellular calcium concentrations. It was observed that in both extracellular calcium conditions higher cell death occurred in HEK-293 cells expressing M163L-Cx26 or D50N-Cx26 in comparison to cells expressing wtCx26 or V84M-Cx26, and no rescue of the increased cell death phenotype associated with M163L and D50N mutations occurred at the high extracellular calcium concentration (Fig. 4A). FACS analysis was also performed to investigate the effect of expressing M163L-Cx26 in the keratinocyte cell line, NEB1 cells, at a physiological extracellular calcium concentration. In NEB1 keratinocytes, cells expressing M163L-Cx26 or D50NCx26 exhibited more cell death than those cells expressing wtCx26 or V84M-Cx26 (Fig. 4B). Cell death was also analysed in HEK-293 cells transfected with M163L-Cx26, wtCx26, and wtCx30 (Fig. 4C). The mutant M163L alone caused the highest cell death. When the mutant was co-transfected with wtCx26 or wtCx30 the cell death rates decreased but were still statistically higher than the ones observed with wtCx26 either alone ($p = 0.036$) or in combination with wtCx30 ($p = 0.011$). Finally, the combination of M163L, wtCx26 and wtCx30 (1:1:2 ratio) resulted in a cell death rate not statistically different ($p = 0.085$) from the one obtained with cells co-transfected equimolarly with wtCx26 and wtCx30, although a small increase could be observed.

4. DISCUSSION

We have identified a novel, apparently dominant, Cx26 mutation, p.M163L, in a Portuguese nuclear family presenting with bilateral NSSHL. This mutation was identified in heterozygosity in two mild/moderate high-frequency hearing impaired individuals (mother and daughter) and was not found in normal hearing controls from the Portuguese population, which suggested it might be a pathogenic variant. This hypothesis was further supported by the

residue conservation analysis, which revealed total conservation of the methionine residue at position 163 across the 23 mammalian species represented at Ensembl and Uniprot databases, and also by reports of another mutation at the same position, M163V, having been found in heterozygosity in hearing loss patients (Marlin *et al*., 2001; Bayazit *et al*., 2003; Dalámon *et al*., 2005). This latter mutation was shown to be translated but failed to induce the formation of homotypic junctional channels (Bruzzone *et al*., 2003).

In order to elucidate the pathogenicity of M163L mutation we performed functional studies regarding its ability to traffic to the cell membrane as well as its effect on cell viability.

The trafficking of M163L-Cx26 to the cell membrane was clearly impaired. However, trafficking to cell membrane with formation of gap junction-like structures was observed when this mutant was co-expressed with either wtCx26 or wtCx30.

As regards cell viability, it was observed that expression of M163L-Cx26 in HEK-293 and NEB1 cells caused an increased cell death when compared to that seen upon expression of wtCx26. Moreover, this phenotype was not rescued by a high extracellular calcium concentration, as it was reported to happen with the G45E mutation, which yielded abnormally open hemi-channels at a physiological range of extracellular calcium concentration, but closed channels at higher concentrations, thus preventing cell death. This result, plus the trafficking defect, suggests that the increased cell death phenotype associated with M163L mutation is not due to abnormal hemi-channels.

It was also observed that the increased cell death associated with the expression of M163L-Cx26 was lowered by equimolar co-expression of wtCx26 or wtCx30. However, co-expression of M163L-Cx26 and wtCx26 still resulted in a higher cell death rate than the one obtained upon transfection with the same amount of total plasmid DNA, solely coding wtCx26. Similarly, equimolar co-expression of M163L-Cx26 and wtCx30 yielded a higher cell death rate than the one observed with cells equimolarly co-expressing wtCx26 and wtCx30. This suggests that the M163L mutant might exert a partially dominant-negative effect on wtCx26 and wtCx30 as regards cell survival.

Previously it has been reported that the second and third rows of Hensen's cells of guinea pigs express Cx26 but almost no Cx30 (Zhao and Yu, 2006). Therefore it is possible that the

viability of cells solely expressing Cx26 in the M163L heterozygous patients might be diminished. As there is a gradient of expression of Cx26 and Cx30 in the cochleae of guinea pigs, with less expression at the base compared to the apex (Zhao and Yu, 2006), it is likely that the putative decreased viability of the patients' Hensen's cells causes more damage to the base of the cochlea, the region responsible for hearing at high-frequencies, than to its apex, the region responsible for hearing at low-frequencies. This hypothesis could explain the high-frequency hearing loss displayed by the patients. Additionally, it is possible that the M163L mutant protein impairs the function of Cx26 and/or Cx26/Cx30 channels, leading to decreased coupling, which also can have more severe consequences in the basal turn than in the apex due to the lower connexin expression.

In conclusion, we have presented functional evidence supporting the pathogenicity and mode of the transmission of the novel dominant Cx26 mutation, p.M163L, therefore contributing to a better understanding of the molecular mechanisms underlying GJB2 related hearing loss.

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REFERENCES

Ballana, E., Ventayol, M., Rabionet, R., Gasparini, P., Estivill, X., 2008. Connexins and deafness Homepage. Available from: World Wide Web URL: <http://www.crg.es/deafness>.

Bayazit, Y.A., Cable, B.B., Cataloluk, O., Kara, C., Chamberlin, P., Smith, R.J.H., Kanlikama, M., Ozer, E., Cakmak, E.A., Mumbuc, S., Arslan, A., 2003. GJB2 gene mutations causing familial hereditary deafness in Turkey. Int. J. Pediatr. Otorhinolaryngol. 67, 1331–1335.

Bruzzone, R., Veronesi, V., Gomès, D., Bicego, M., Duval, N., Marlin, S., Petit, C., D'Andrea, P., White, T.W., 2003. Loss-of-function and residual channel activity of connexin26 mutations associated with non-syndromic deafness. FEBS Lett. 533, 79–88.

Clamp, M., Cuff, J., Searle, S.M., Barton, G.J., 2004. The Jalview Java alignment editor. Bioinformatics 20, 426–427.

Common, J.E.A., Di, W.-L., Davies, D., Kelsell, D.P., 2004. Further evidence for heterozygote advantage of GJB2 deafness mutations: a link with cell survival. J. Med. Genet. 41, 573–575.

Cooper, D.N., Ball, E.V., Stenson, P.D., Phillips, A.D., Howells, K., Mort, M.E., 2007. The Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff. Available from: <http://www.hgmd.cf.ac.uk/ac/index.php>.

Dalámon, V., Béhèran, A., Diamante, F., Pallares, N., Diamante, V., Elgoyhen, A.B., 2005. Prevalence of GJB2 mutations and the del(GJB6-D13S1830) in Argentinean non-syndromic deaf patients. Hear. Res. 207 (1-2), 43–49.

Del Castillo, F.J., Rodríguez-Ballesteros, M., Álvarez, A., Hutchin, T., Leonardi, E., de Oliveira, C.A., Azaiez, H., Brownstein, Z., Avenarius, M.R., Marlin, S., Pandya, A., Shahin, H., Siemering, K.R., Weil, D., Wuyts, W., Aguirre, L.A., Martı´n, Y., Moreno-Pelayo, M.A., Villamar, M., Avraham, K.B., Dahl, H.M., Kanaan, M., Nance, W.E., Petit, C., Smith, R.J.H., Van Camp, G., Sartorato, E.L., Murgia, A., Moreno, F., del Castillo, I., 2005. A novel deletion involving the connexin-30 gene, del(GJB6-d13s1854), found in trans with mutations in the GJB2 gene (connexin-26) in subjects with DFNB1 non-syndromic hearing impairment. J. Med. Genet. 42, 588–594.

Di, W.-L., Gu, Y., Common, J.E.A., Aasen, T., O'Toole, E.A., Kelsell, D.P., Zicha, D., 2005.Connexin interaction patterns in keratinocytes revealed morphologically and by FRET analysis. J. Cell Sci. 118, 1505–1514.

Estivill, X., Govea, N., Barceló, E., Badenas, C., Romero, E., Moral, L., Scozzri, R., D'Urbano, L., Zeviani, M., Torroni, A., 1998. Familial progressive sensorineural deafness is mainly due to the mtDNA A1555G mutation and is enhanced by treatment of aminoglycosides. Am. J. Hum. Genet. 62, 27–35.

Fischel-Ghodsian, N., Prezant, T.R., Fournier, P., Stewart, I.A., Maw, M., 1995. Mitochondrial mutation associated with non-syndromic deafness. Am. J. Otolaryngol. 16, 403–408.

Forge, A., Becker, D., Casalotti, S., Edwards, J., Marziano, N., Nevill, G., 2003. Gap junctions in the inner ear: comparison of distribution patterns in different vertebrates and assessment of connexin composition in mammals. J. Comp. Neurol. 467, 207–231.

Marlin, S., Garabédian, E.N., Roger, G., Moatti, L., Matha, N., Lewin, P., Petit, C., Denoyelle, F., 2001. Connexin 26 gene mutations in congenitally deaf children: pitfalls for genetic counseling. Arch. Otolaryngol. Head Neck Surg. 127 (8), 927–933.

Marziano, N.K., Casalotti, S.O., Portelli, A.E., Becker, D.L., Forge, A., 2003. Mutations in the gene for connexin 26 (GJB2) that cause hearing loss have a dominant negative effect on connexin 30. Hum. Mol. Genet. 12, 805–812.

Matos, T.D., Caria, H., Simões-Teixeira, H., Aasen, T., Nickel, R., Jagger, D., O'Neill, A., Kelsell, D., Fialho, G., 2007. A novel hearing-loss-related mutation occurring in the GJB2 basal promoter. J. Med. Genet. 44, 721–725.

Scott, D.A., Kraft, M.L., Carmi, R., Ramesh, A., Elbedour, K., Yairi, Y., Srisailapathy, C.R., Rosengren, S.S., Markham, A.F., Mueller, R.F., Lench, N.J., Van Camp, G., Smith, R.J., Sheffield, V.C., 1998.

Identification of mutations in the connexin 26 gene that cause autosomal recessive nonsyndromic hearing loss. Hum. Mutat. 11, 387–394.

Stong, B.C., Chang, Q., Ahmad, S., Lin, X., 2006. A novel mechanism for connexin 26 mutation linked deafness: cell death caused by leaky gap junction hemichannels. Laryngoscope 116, 2205–2210.

Wangemann, P., 2002. K^+ cycling and the endocochlear potential. Hear. Res. 165, 1–9.

Zhao, H., Yu, N., 2006. Distinct and gradient distributions of connexin26 and connexin30 in the cochlear sensory epithelium of guinea pigs. J. Comp. Neurol. 499, 506–518.

FIGURES

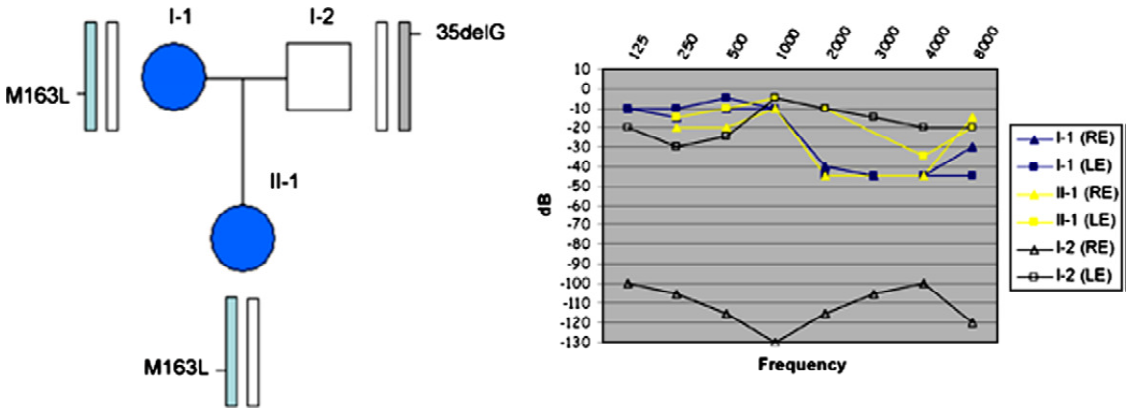

Figure 1. Pedigree and audiograms of the members of the family. A dominant form of NSSHL is apparently segregating in this family, affecting mother (I-1) and daughter (II-1), who are represented in the pedigree by blue symbols. These individuals have similar audiogram shapes and similar degrees of hearing loss. The slightly milder degree of hearing loss in the daughter might indicate either progressive deafness or intra-familial variability of the phenotype. I-2 presents with unilateral profound hearing loss apparently due to chronic otitis.

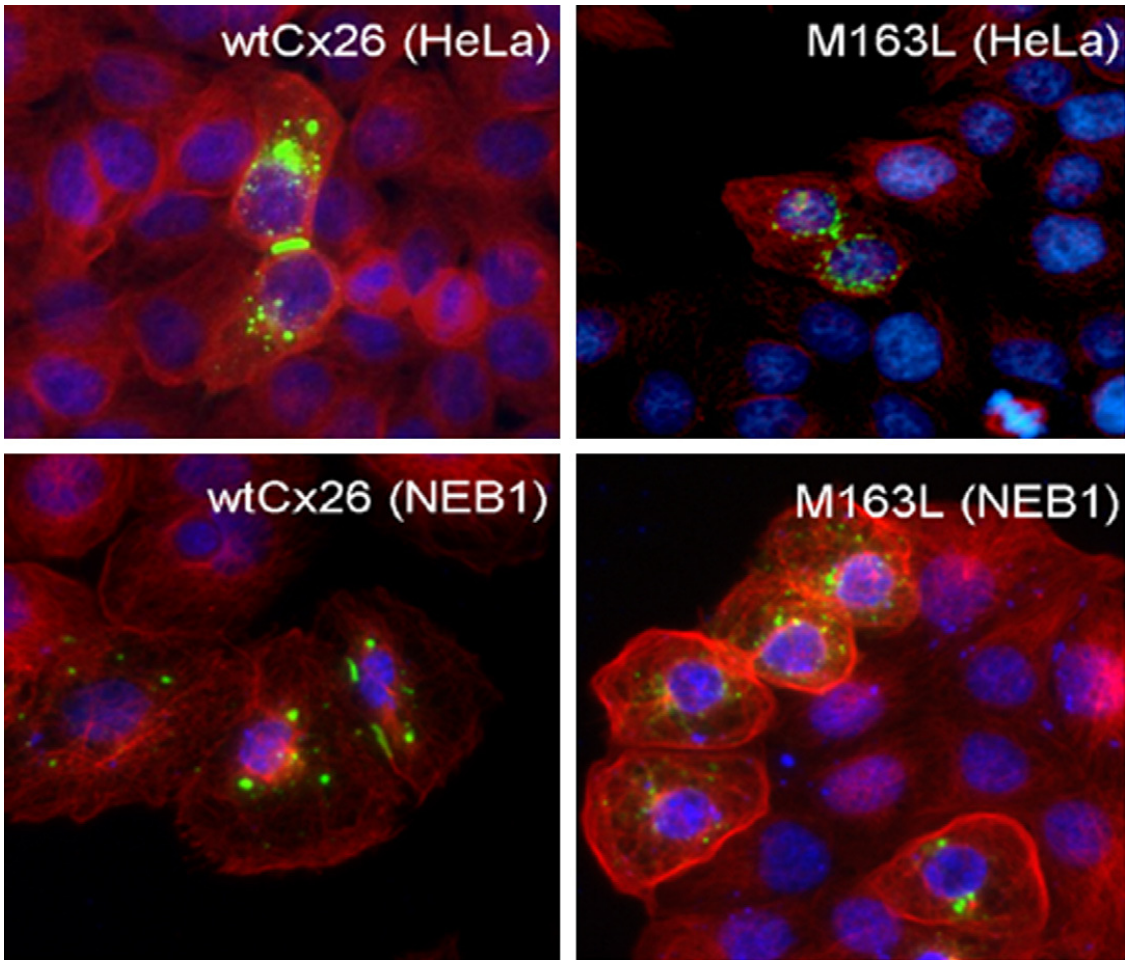

Figure 2. Microscopy images showing the cellular localization of wtCx26 and M163L-Cx26 proteins in HeLa and NEB1 cells. Wild-type Cx26 traffics to cell membrane, forming gap junction-like aggregates of protein. M163L-Cx26 does not traffic to cell membrane, being only visible in the cytoplasm. Note, also, the rounded, detaching morphology of NEB1 cells expressing the M163L mutant protein.

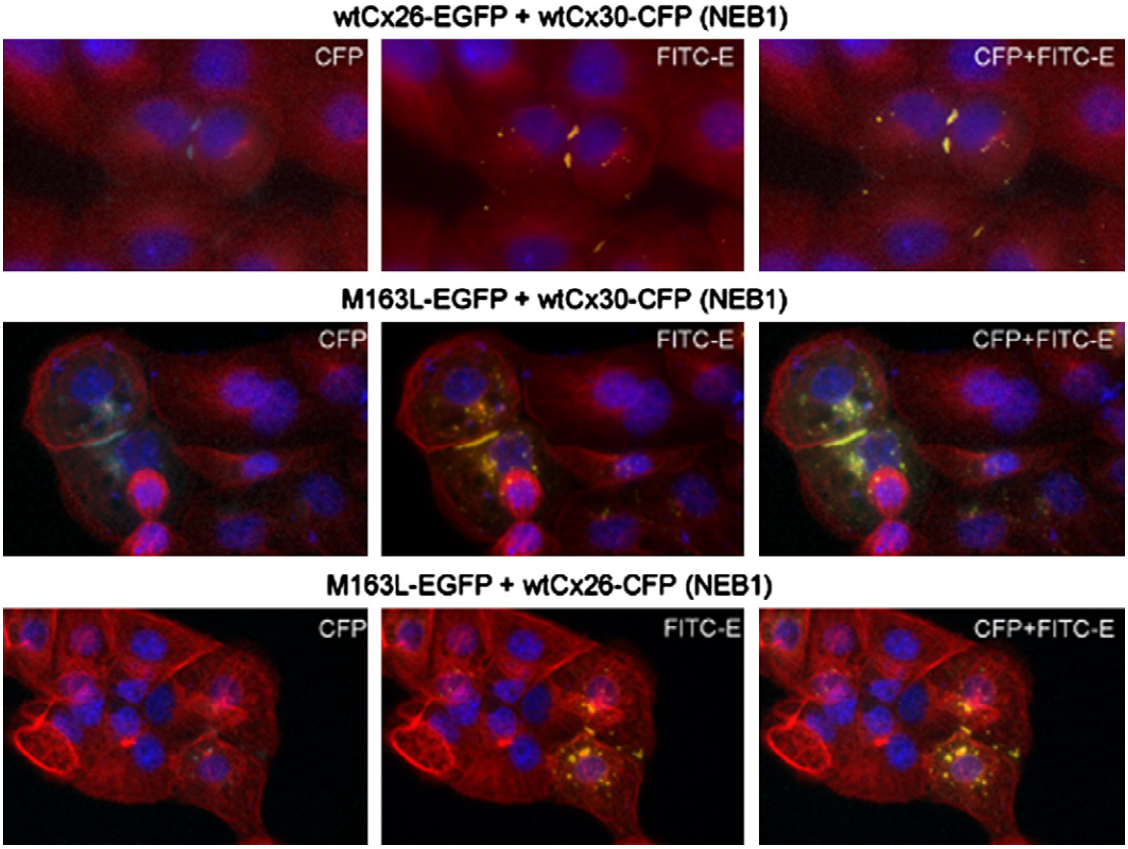

Figure 3. Microscopy images showing the co-localisation of M163L-Cx26 with either wtCx26 or wtCx30, at gap junction-like structures in NEB-1 cells.

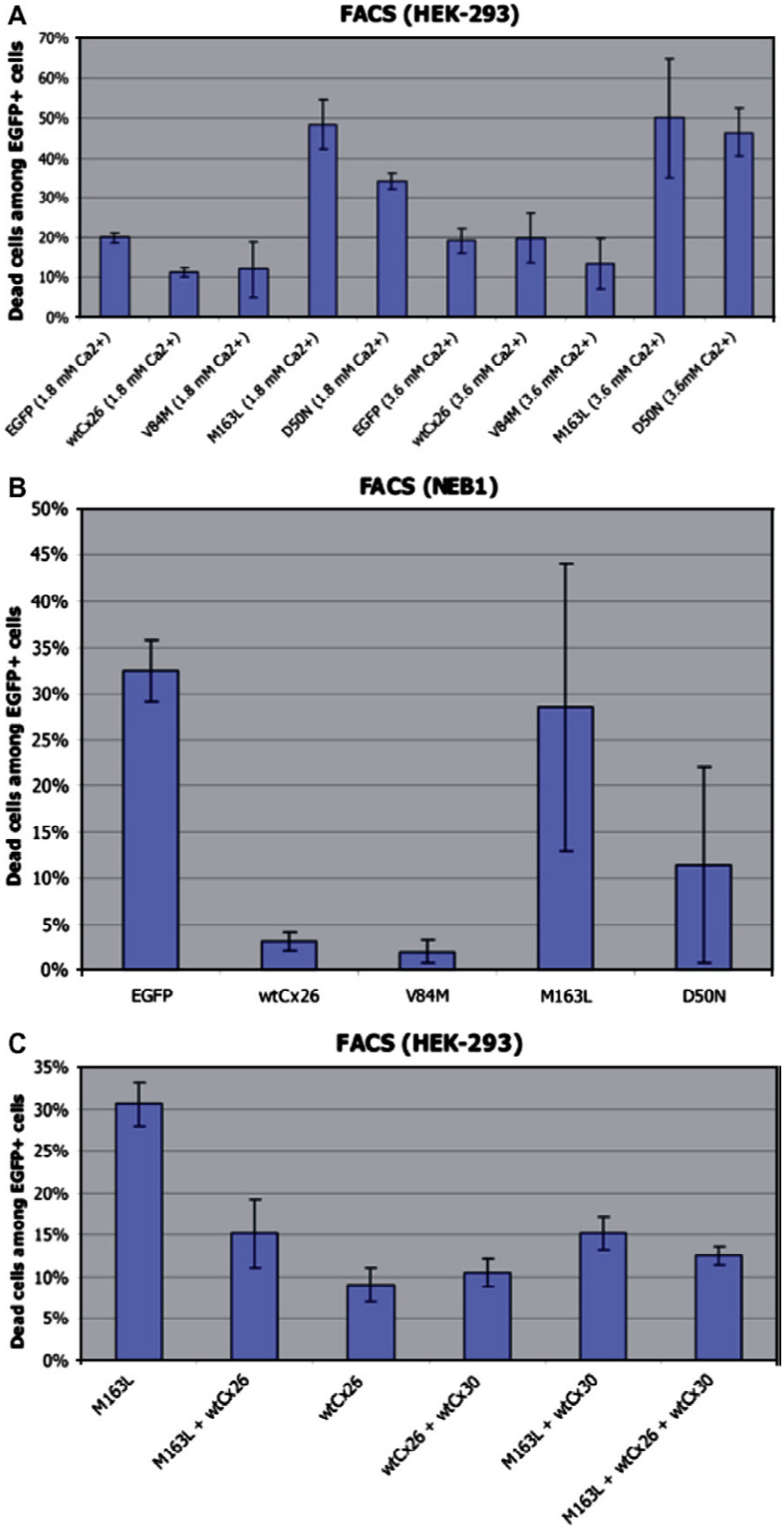
A
FACS (HEK-293)
Dead cells among EGFP+ cells
70%
60%
50%
40%
30%
20%
10%
0%
EGFP (1.8 mM Ca2+)
wtCx26 (1.8 mM Ca2+)
V84M (1.8 mM Ca2+)
M163L (1.8 mM Ca2+)
D50N (1.8 mM Ca2+)
EGFP (3.6 mM Ca2+)
wtCx26 (3.6 mM Ca2+)
V84M (3.6 mM Ca2+)
M163L (3.6 mM Ca2+)
D50N (3.6mM Ca2+)
B
FACS (NEB1)
Dead cells among EGFP+ cells
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
EGFP
wtCx26
V84M
M163L
D50N
C
FACS (HEK-293)
Dead cells among EGFP+ cells
35%
30%
25%
20%
15%
10%
5%
0%
M163L
M163L + wtCx26
wtCx26
wtCx26 + wtCx30
M163L + wtCx30
M163L + wtCx26 + wtCx30

Figure 4. (A) FACS analysis of HEK-293 cells at a physiological (1.8 mM) and at a high (3.6 mM) extracellular calcium concentrations. (B) FACS analysis of NEB1 cells at a physiological extracellular calcium concentration (1.05 mM). (C) FACS analysis of HEK-293 cells at a physiological extracellular calcium concentration (1.8 mM).

SUPPLEMENTARY INFORMATION

Materials and Methods

Cell Culture

See Supplementary Information in Chapter 3.

Western Blot

HeLa cells, previously transfected with pEGFP-N3 constructs encoding wtCx26, p.Val84Met-Cx26 and p.Met163Leu-Cx26 were washed twice with PBS, and then lysed in lysis solution (5% 2-Mercaptoethanol in SDS sample buffer with protease inhibitor) with the aid of a syringe plunger. The lysates were sonicated to reduce viscosity. The samples were loaded into 10% polyacrylamide gels, and the protein size marker used was the Full-Range Rainbow Molecular Weight Marker (GE Wealthcare). The samples were run, at 110V, until the loading buffer had just got off the gel. The proteins in the gel were then transferred to a membrane (Amersham Hybond™ ECL™). After the transfer, the membrane was blocked in PBS with 5% milk powder. The membrane was hybridized with the primary antibody (GFP antibody ab290, Abcam), diluted 1:1000 in PBS with 5% milk powder, during 1h at room temperature, with shaking, and then, after three 5min washes in PBS 1% Tween, it was incubated with the secondary antibody (goat anti-rabbit HRP P0448, DAKO) in the same conditions. Following three washes with PBS-1% Tween, the membrane was developed using the Amersham ECL™ Western Blotting Detection Reagents. After development, the membrane was stripped and hybridized with a mixture of antibodies against GAPDH (ab8245, Abcam) and β-actin (A5441, Sigma) first, and then with a goat anti-mouse secondary antibody (1:1000) and was revealed.

ANNEX

Figures

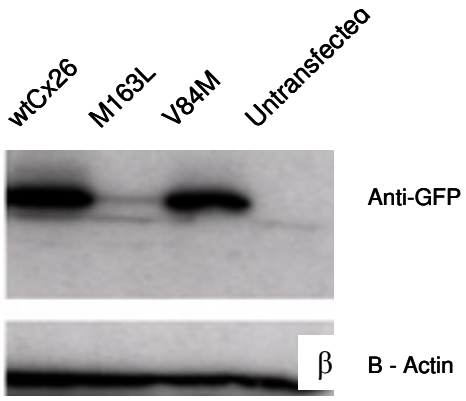

Figure 1. Western Blot of HeLa whole cell lysates revealed similar wild-type and p.Val84Met-Cx26 (V84M) protein amounts, but only a small amount of p.Met163Leu-Cx26 (M163L) protein was detected.

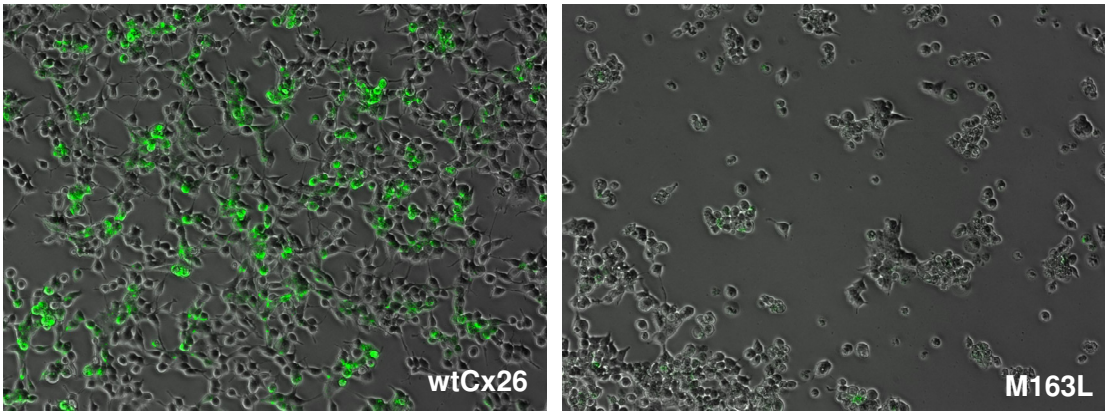

Figure 2. HEK-293 transfected with either wtCx26 or p.Met163Leu-Cx26 (M163L). Expression of the p.Met163Leu-Cx26 protein led to increased cell death.

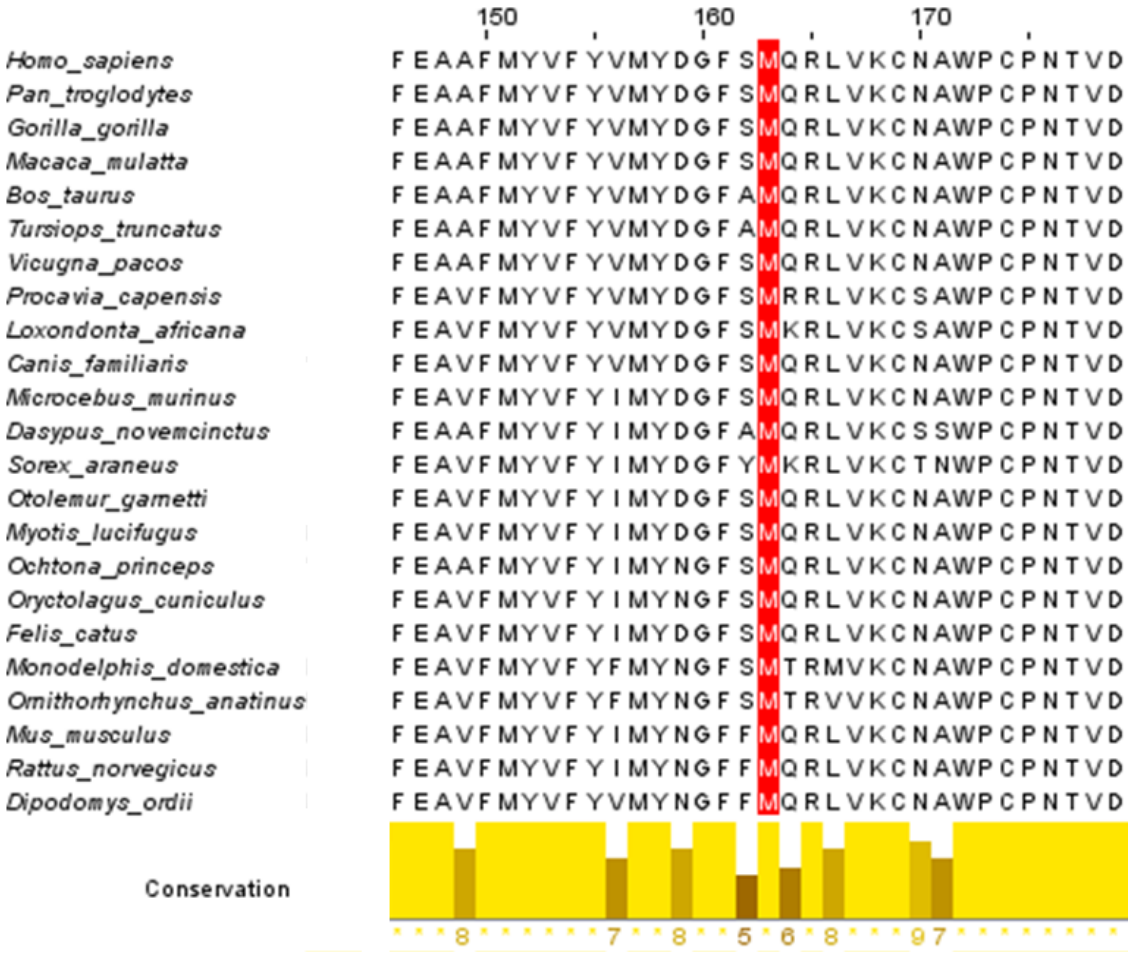

Figure 3. Multiple alignment of the Cx26 sequences from 23 mammalian species. The sequences were derived from the Ensembl database (Flicek *et al*., 2011). The multiple alignment was performed in ClustalW 2.1 (Larkin *et al*., 2007) and visualized in JalView 2.6.1 (Waterhouse, Procter, Martin, Clamp, & Barton, 2009). The methionine at position 163 (highlighted in red) is invariant across the aligned sequences. This multiple alignment serves to illustrate the conservation of the amino acid residue at position 163 of Cx26, mentioned in the article of this chapter, but it is not the alignment mentioned in the article, as it was done by using sequences derived from the Ensembl database only.

References

Flicek, P., Amode, M. R., Barrell, D., Beal, K., Brent, S., Chen, Y., Clapham, P., *et al*. (2011). Ensembl 2011. *Nucleic Acids Research*, *39*(Database issue), D800-D806.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., *et al*. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, *23*(21), 2947-2948.

Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M., & Barton, G. J. (2009). Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics*, *25*(9), 1189-1191.

CHAPTER 5

The controversial p.Arg127His mutation in GJB2

The Controversial p.Arg127His Mutation in *GJB2*: Report on Three Portuguese Hearing Loss Family Cases

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ABSTRACT

Mutations in the *GJB2* gene account for up to 50% of hereditary nonsyndromic hearing loss in several populations. Over 200 mutations are already described in this gene, and three of them, c.35delG, c.167delT, and c.235delC, are the most frequent in Caucasians, Ashkenazi Jews, and Asians, respectively. Most of *GJB2* hearing loss-related mutations are recessive, but a few dominant alleles have also been described. Apart from the clearly pathogenic mutations, there are some other variants whose pathogenicity is still controversial, such as p.Met34Thr, p.Val37Ile, p.Arg127His, and p.Val153Ile. The p.Arg127His allele has been found in some mono- and biallelic hearing-impaired patients from several countries. In this article we report on some Portuguese patients harboring this mutation. Taking into consideration the analysis of these Portuguese cases as well as the genetic and functional data regarding p.Arg127His available in the literature, we conclude that this variant may be a cause of hearing loss depending on environmental factors and/or genetic background.

INTRODUCTION

Genetic hearing loss is a common sensorial disorder in humans. There are many genes that, when mutated, can cause this disease. However, mutations in the gene encoding the gap junction protein connexin 26 (*GJB2*) account for up to 50% of hereditary nonsyndromic hearing loss in several populations. The Human Genome Mutation Database contains over 200 mutations described in the *GJB2* gene, most of them considered pathogenic (Stenson *et al*., 2009) (www.hgmd.org). However, there are some variants, such as p.Met34Thr, p.Val37Ile, p.Arg127His, and p.Val153Ile, whose pathogenicity is still controversial, since their role in hearing impairment is not yet clearly understood (Marlin *et al*., 2001; Wu *et al*., 2002; Bayazit *et al*., 2003; Meşe *et al*., 2004; Bicego *et al*., 2006; Huculak *et al*., 2006; Guerci *et al*., 2007; Pollak *et al*., 2007).

Here we report on three Portuguese families affected with hearing loss, in which we have identified p.Arg127His. This variant is common in both deaf and hearing Indians, presenting allele frequencies of 0.123 and 0.175, respectively, in the study by RamShankar *et al*. (2003). Similar frequencies were reported by Ramchander *et al*. (2004) in a different sample of Indian patients and hearing controls. The p.Arg127His variant was also identified in 19.4% of the chromosomes of 54 hearing-impaired Slovak Romany (Gypsies), a people with Indian ancestry (Minárik *et al*., 2003; Álvarez *et al*., 2005). Interestingly, two of the families here presented belong to the Gypsy community. We discuss the pathogenicity of p.Arg127His taking into consideration our results as well as the information available in the literature on genetic and functional aspects of this mutation. In this way, this work may contribute to a better understanding of the role of p.Arg127His mutation in hearing loss.

MATERIALS AND METHODS

Patients

This study focuses on three Portuguese families (coded as BS, DM, and EL), presenting with bilateral nonsyndromic, sensorineural hearing loss, whose analysis was performed when screening Portuguese affected families for *GJB2* mutations. All patients were audiologically evaluated by pure tone audiometry. Informed consent was obtained from all the participants.

Genetic analysis

Blood samples were taken from the participants, and genomic DNA was extracted. The *GJB2* gene coding region and acceptor splice site was amplified, and sequenced using the newly designed forward primer 2aF 5'-AAGTCTCCCTG TTCTGTCCT-3' and reverse primer 2bR 5'-GGCATCTGGAGTTTCACC-30. All the patients, except I:2 from family DM, were further studied in regard to the del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) *GJB6* deletions using the same methodology as del Castillo *et al*. (2005). Afterward, these individuals were also screened by

sequencing for mutations in the *GJB2* basal promoter, exon 1, and donor splice site, according to Matos *et al*. (2007).

RESULTS

The hearing-impaired individuals belonging to three Portuguese families were analyzed in respect to *GJB2* coding region and acceptor splice site. With the exception of individual I:2 belonging to family DM, these patients were also tested for both the common *GJB6* deletions and screened for mutations in the *GJB2* basal promoter, exon 1, and donor splice site. The variant p.Arg127His (R127H) was found in the three families analyzed, but in two of them a second *GJB2* mutation, p.Trp24X (W24X) or c.-23+1G>A (often referred as IVS1+1G>A), was also identified (Fig. 1). No further *GJB2* mutation, or *GJB6* deletion, was found.

In one of the families (EL), of Gypsy ethnicity, the proband, his affected sibling, and the normal-hearing father are compound heterozygous for p.Trp24X/p.Arg127His; the normal-hearing mother is homozygous for p.Arg127His. In the second family (DM), consanguineous and also of Gypsy ethnicity, the p.Arg127His mutation does not seem to segregate with hearing loss since the two affected siblings are heterozygous for p.Arg127His, with no accompanying *GJB2* mutation or *GJB6* deletion, while their unaffected sibling is homozygous for this mutation. In the third family (BS), of unknown ethnicity, the proband is heterozygous for p.Arg127His as well as for the recessive, donor splice-site mutation c.-23+1G>A. Genetic analysis on the parents' DNA was performed to determine whether the two mutations are in *trans*. It was observed that each parent is a carrier of one of the mutations, so the child is effectively compound heterozygous.

DISCUSSION

We have analyzed three Portuguese families (BS, DM, and EL) presenting with sensorineural hearing loss, and having in common the controversial p.Arg127His mutation in

GJB2. However, this variant does not seem to be a cause of hearing impairment in all the three families.

In family DM, the results obtained suggest that the hearing loss is not likely to be related to the p.Arg127His mutation since both affected siblings are heterozygous for this variant, with no accompanying *GJB2* mutation or *GJB6* deletion, while the normal-hearing sibling is homozygous for p.Arg127His. The cause of the hearing loss might probably lie in recessive alleles of other genes brought together by consanguinity.

In the other two families, BS and EL, the p.Arg127His mutation could be involved in the hearing loss observed, since it occurs in compound heterozygosity with two well established recessive mutations, p.Trp24X and c.-23+1G>A, in the affected individuals (although other gene or environmental factors cannot be excluded as causes for the hearing loss). In family BS, the p.Arg127His variant seems to behave as a recessive allele since the proband is a c.-23+1G>A/p.Arg127His compound heterozygote, and the parents, each one a carrier of one of the mutations, have normal hearing. In family EL, the proband and his affected sibling are both compound heterozygous for p.Trp24X and p.Arg127His, a similar situation to the one observed in a hearing loss case reported by Roux *et al*. (2004). The mutation p.Arg127His has also been found, in other cases of hearing loss, associated with the pathogenic recessive mutation p.Glu47Lys (Prasad *et al*., 2000), and also with the controversial variants p.Met34Thr (Roux *et al*., 2004; Snoeckx *et al*., 2005), p.Val37Ile (Yaeger *et al*., 2006) and p.Val153Ile (Najmabadi *et al*., 2005, Santos *et al*., 2005). Noteworthy, in the study by Roux *et al*. (2004), the three p.Arg127His alleles identified in the patient cohort occurred in compound heterozygosity with p.Met34Thr (two probands) and with p.Trp24X (one proband), while in the general population no compound heterozygosity involving p.Arg127His was reported. The authors considered significant the fact that compound heterozygosity for p.Met34Thr and p.Arg127His - identified twice among the patients - had not been observed in the general population controls. In other study (Tóth *et al*., 2004), p.Arg127His was shown to segregate in homozygosity with hearing loss. All these findings would support the pathogenicity of the p.Arg127His variant.

However, some observations reported in the literature seem to argue against the pathogenic nature of p.Arg127His. In family EL and in the case described by Roux *et al*. (2004)

mentioned above, compound heterozygosity for p.Trp24X and p.Arg127His was also observed in normal-hearing relatives. A puzzling behavior of the p.Arg127His variant is again evident when considering the reports of normal-hearing individuals (Marlin *et al*., 2001) and hearing-impaired individuals (Uyguner *et al*., 2003) who are compound heterozygotes for the c.35delG and p.Arg127His mutations. Moreover, normal-hearing individuals homozygous for p.Arg127His have been reported (RamShankar *et al*., 2003; Roux *et al*., 2004; Dahl *et al*., 2006), and studies have been published in which the frequency of the p.Arg127His variant in hearing-impaired and normal-hearing individuals is not significantly different, in comparison to clearly pathogenic mutations such as p.Trp24X and c.35delG (RamShankar *et al*., 2003; Ramchander *et al*., 2004; Roux *et al*., 2004).

Given the discrepancies observed in the genetic studies, investigating the functionality of the p.Arg127His-Cx26 protein could provide further insight into the role of this variant in hearing loss. With this objective, some functional studies were already performed by other groups.

Palmada *et al*. (2006) investigated the role of p.Arg127His mutation on hemichannel conductance in noncoupled oocytes. Western blotting ensured that p.Arg127His-Cx26 was inserted in the oocytes' membranes at a degree similar to wtCx26. In the subsequent measurements of hemichannel activity, p.Arg127His-Cx26 displayed a partially defective phenotype when expressed alone. Although coexpression of p.Arg127His-Cx26 with wtCx26 at equimolar levels (mimicking p.Arg127His heterozygosity) revealed no significant current difference relative to wtCx26 expressed alone, coexpression of p.Arg127His-Cx26 with wtCx30 or wtCx31 at equimolar levels (mimicking p.Arg127His homozygosity) revealed a strong dominant negative effect of the p.Arg127 His-Cx26 mutant on each of the wild-type connexins.

Wang *et al*. (2003) performed dual whole-cell voltage-clamp assays in N2A cells stably transfected with cDNA encoding p.Arg127His-Cx26, and demonstrated that transjunctional current and macroscopic junctional conductance of p.Arg127 His-Cx26 channels were greatly reduced. The authors also reported impaired neurobiotin transfer between pairs of N2A cells expressing p.Arg127His-Cx26 (n=1 out of six pairs). These results seem not to be due to impaired translation or protein trafficking to the membrane, since Western blot revealed strong

expression of the p.Arg127His-Cx26, and this mutant protein was predominantly expressed in the cell membrane being its immunocytochemical staining pattern indistinguishable from that of wtCx26. Bicego *et al*. (2006) and D'Andrea *et al*. (2002) also observed immunocalization of p.Arg127His-Cx26 similar to wtCx26, with junctional plaques in zones of cell-to-cell apposition, and Western blotting revealed that levels of p.Arg127His-Cx26 expression were comparable (small or no significant difference) with those of wtCx26. In these two studies, p.Arg127His-Cx26 channels have been found to have a much reduced permeability to Lucifer yellow, but p.Arg127His-Cx26 did not show a dominant negative effect on wtCx26, regarding permeability to this tracer, when both variants were cotransfected at 1:1 ratio. p.Arg127His-Cx26 channels were also shown to fail diamidino-2-phenylindole (DAPI) transfer (D'Andrea *et al*., 2002).

Thönnissen *et al*. (2002) observed an elevated permeability of p.Arg127His-Cx26 channels to neurobiotin, contrary to the results obtained by Wang *et al*. (2003) above referred. It should, however, be noticed that in the study by Thönnissen *et al*. (2002), p.Arg127His-Cx26 was considerably more expressed than wtCx26; therefore, the high intercellular diffusion of neurobiotin observed with p.Arg127His-Cx26 channels (three times more than that obtained with wtCx26 channels) might not reflect the real permeability of each p.Arg127His-Cx26 channel; instead, it may be due to the very high number of p.Arg127His-Cx26 channels with reduced permeability to neurobiotin, as suggested by the results of Wang *et al*. (2003). This notoriously higher expression of p.Arg127His-Cx26 relative to wtCx26, which was not observed by D'Andrea *et al*. (2002) or by Bicego *et al*. (2006), might result from a different degree of purity of the constructs used for transfection, or it may have happened that the stably transfected p.Arg127His-Cx26 expressing clones chosen for subsequent analyses expressed, by chance, more Cx26 protein than the ones expressing wtCx26. It is also possible that the silent base change, from G to A, that the authors refer to exist at position 237 in the Cx26 sequence in all the constructs used might have altered protein expression levels in a manner that changed the relative expression of p.Arg127His-Cx26 and wtCx26.

In summary, the overall results clearly indicate an abnormal behavior of this mutation.

It is not known whether the alterations in the properties of p.Arg127His-Cx26 channels observed *in vitro* are meaningful at the biological level and, if so, at what extent or under which

circumstances. If any *in vivo* effect exists, it might be potentiated or attenuated by other genetic and/or environmental factors. These still unknown interactions would explain the differences observed in the audiological phenotypes (normal or impaired) of the compound heterozygotes for p.Arg127His and p.Trp24X in family EL and in the family referred by Roux *et al*. (2004). This explanation could be extended to other cases of compound heterozygosity involving p.Arg127His and to the p.Arg127His homozygotes.

In the light of the genetic and functional data here analyzed, including the three Portuguese cases, we conclude that p.Arg127His mutation may in some cases be a cause of hearing loss, depending on environmental and/or genetic factors yet to be elucidated. Therefore, p.Arg127His should not be disregarded when providing genetic counseling to the families.

ACKNOWLEDGEMENTS

We would like to thank the family members for their collaboration. This study was supported by Fundação para a Ciência e a Tecnologia grants SFRH/BD/19988/2004 (T.D.M.) and SFRH/BD/24575/2005 (H.S.-T.).

REFERENCES

Álvarez A, del Castillo I, Villamar M, *et al*. (2005) High prevalence of the W24X mutation in the gene encoding connexin-26 (GJB2) in Spanish Romani (Gypsies) with autosomal recessive non-syndromic hearing loss. Am J Med Genet A 137A:255–258.

Bayazit YA, Cable BB, Cataloluk O, *et al*. (2003) GJB2 gene mutations causing familial hereditary deafness in Turkey. Int J Pediatr Otorhinolaryngol 67:1331–1335.

Bicego M, Beltramello M, Melchionda S, *et al*. (2006) Pathogenetic role of the deafness-related M34T mutation of Cx26. Hum Mol Genet 15:2569–2587.

Dahl HH, Tobin SE, Poulakis Z, *et al*. (2006) The contribution of GJB2 mutations to slight or mild hearing loss in Australian elementary school children. J Med Genet 43:850–855.

D'Andrea P, Veronesi V, Bicego M, *et al*. (2002) Hearing loss: frequency and functional studies of the most common connexin26 alleles. Biochem Biophys Res Commun 296:685–691.

del Castillo FJ, Rodríguez-Ballesteros M, Alvarez A, *et al*. (2005) A novel deletion involving the connexin-30 gene, del(GJB6-d13s1854), found in trans with mutations in the GJB2 gene (connexin-26) in subjects with DFNB1 nonsyndromic hearing impairment. J Med Genet 42:588–594.

Guerci VI, Grasso DL, Morgutti M, *et al*. (2007) Connexin 26 gene: defining the role of the V153I mutation. Audiol Med 5:200–206.

Huculak C, Bruyere H, Nelson TN, *et al*. (2006) V37I connexin 26 allele in patients with sensorineural hearing loss: evidence of its pathogenicity. Am J Med Genet A 140A:2394–2400.

Marlin S, Garabédian EN, Roger G, *et al*. (2001) Connexin 26 gene mutations in congenitally deaf children: pitfalls for genetic counseling. Arch Otolaryngol Head Neck Surg 127:927–933.

Matos TD, Caria H, Simões-Teixeira H, *et al*. (2007) A novel hearing loss related mutation occurring in the GJB2 basal promoter. J Med Genet 44:721–725.

Meşe G, Londin E, Mui R, *et al*. (2004) Altered gating properties of functional Cx26 mutants associated with recessive nonsyndromic hearing loss. Hum Genet 115:191–199.

Minárik G, Ferák V, Feráková E, *et al*. (2003) High frequency of GJB2 mutation W24X among Slovak Romany (Gypsy) patients with non-syndromic hearing loss (NSHL). Gen Physiol Biophys 22:549–556.

Najmabadi H, Nishimura C, Kahrizi K, *et al*. (2005) GJB2 mutations: passage through Iran. Am J Med Genet A 133:132–137.

Palmada M, Schmalisch K, Böhmer C, *et al*. (2006) Loss of function mutations of the GJB2 gene detected in patients with DFNB1-associated hearing impairment. Neurobiol Dis 22:112–118.

Pollak A, Skórka A, Mueller-Malesińska M, *et al*. (2007) M34T and V37I mutations in GJB2 associated hearing impairment: evidence for pathogenicity and reduced penetrance. Am J Med Genet A 143A:2534–2543.

Prasad S, Cucci RA, Green GE, *et al*. (2000) Genetic testing for hereditary hearing loss: connexin 26 (GJB2) allele variants and two novel deafness-causing mutations (R32C and 645-648delTAGA). Hum Mutat 16:502–508.

Ramchander PV, Nandur VU, Dwarakanath K, *et al*. (2004) Study of families of nonsyndromic hearing impairment segregating with mutations in Cx26 gene. Indian J Hum Genet 10:58–64.

RamShankar M, Girirajan S, Dagan O, *et al*. (2003) Contribution of connexin26 (GJB2) mutations and founder effect to nonsyndromic hearing loss in India. J Med Genet 40:e68.

Roux AF, Pallares-Ruiz N, Vielle A, *et al*. (2004) Molecular epidemiology of DFNB1 deafness in France. BMC Med Genet 5:5 [Abstract].

Santos RL, Wajid M, Pham TL, *et al*. (2005) Low prevalence of connexin 26 (GJB2) variants in Pakistani families with autosomal recessive non-syndromic hearing impairment. Clin Genet 67:61–68.

Snoeckx RL, Huygen PL, Feldmann D, *et al*. (2005) GJB2 mutations and degree of hearing loss: a multicenter study. Am J Hum Genet 77:945–957.

Stenson PD, Mort M, Ball EV, *et al*. (2009) The Human Gene Mutation Database (HGMD®): 2008 update. Genome Med 1:13.1–13.6.

Thönnissen E, Rabionet R, Arbonès ML, *et al*. (2002) Human connexin26 (GJB2) deafness mutations affect the function of gap junction channels at different levels of protein expression. Hum Genet 111:190–197.

Tóth T, Kupka S, Haack B, *et al*. (2004) GJB2 mutations in patients with nonsyndromic hearing loss from Northeastern Hungary. Hum Mutat 23:631–632.

Uyguner O, Emiroglu M, Uzumcu A, *et al*. (2003) Frequencies of gap- and tight-junction mutations in Turkish families with autosomal-recessive nonsyndromic hearing loss. Clin Genet 64:65–69.

Wang HL, Chang WT, Li AH, *et al*. (2003) Functional analysis of connexin-26 mutants associated with hereditary recessive deafness. J Neurochem 84:735–742.

Wu BL, Lindeman N, Lip V, *et al*. (2002) Effectiveness of sequencing connexin 26 (GJB2) in cases of familial or sporadic childhood deafness referred for molecular diagnostic testing. Genet Med 4:279–288.

Yaeger D, McCallum J, Lewis K, *et al*. (2006) Outcomes of clinical examination and genetic testing of 500 individuals with hearing loss evaluated through a genetics of hearing loss clinic. Am J Med Genet A 140:827–836.

FIGURES

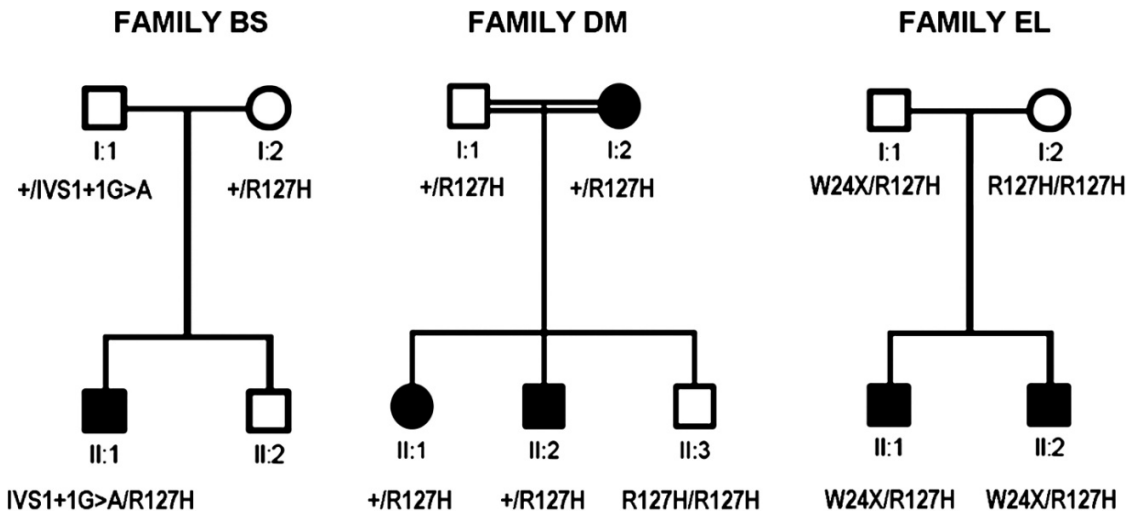

Figure 1. Pedigrees of the three Portuguese families under study, family BS, family DM, and family EL. Black symbols represent hearing-impaired individuals, and open symbols represent normal-hearing individuals. W24X and R127H are abbreviations for the p.Trp24X and p.Arg127His mutations, respectively.

CHAPTER 6

Noncoding regions of GJB2

Assessing noncoding sequence variants of *GJB2* for hearing loss association

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ABSTRACT

Involvement of *GJB2* noncoding regions in hearing loss (HL) has not been extensively investigated. However, three noncoding mutations, c.-259C>T, c.-23G>T, and c.-23+1G>A, were reported. Also, c.-684_-675del, of uncertain pathogenicity, was found upstream of the basal promoter. We performed a detailed analysis of *GJB2* noncoding regions in Portuguese HL patients (previously screened for *GJB2* coding mutations and the common *GJB6* deletions) and in control subjects, by sequencing the basal promoter and flanking upstream region, exon 1, and 3'UTR. All individuals were genotyped for c.-684_-675del and 14 SNPs. Novel variants (c.-731C>T, c.-26G>T, c.*45G>A, and c.*985A>T) were found in controls. A hearing individual homozygous for c.-684_-675del was for the first time identified, supporting the nonpathogenicity of this deletion. Our data indicate linkage disequilibrium (LD) between SNPs rs55704559 (c.*168A>G) and rs5030700 (c.*931C>T) and suggest the association of c.[*168G;*931T] allele with HL. The c.*168A>G change, predicted to alter mRNA folding, might be involved in HL.

1. INTRODUCTION

About two hundred *GJB2* mutations causing nonsyndromic hearing loss (NSHL) have been reported (http://www.hgmd.org/) [1]. Most *GJB2* mutations described so far localize to the coding region (totally included within exon 2), which is routinely analysed upon the study of *GJB2* in HL patients. Also involved in HL, two deletions, del(*GJB6*-D13S1830) [2–4] and del(*GJB6*-D13S1854) [5] disrupt the *GJB6* gene which codes for connexin-30, and it is thought that they may ablate a *GJB2 cis*-regulatory sequence [5-7]. This putative element is likely to be ablated by a third deletion, del(chr13 : 19,837,344–19,968,698), localized upstream of *GJB2* and *GJB6* [8, 9].

Along with *GJB2* coding region, the noncoding first exon and donor splice site have been analysed in several studies, and two pathogenic mutations, c.-23G>T (exon 1) [10] and c.-23+1G>A (intron) [11], both in the donor splice site, have been identified. The c.-23+1G>A mutation (commonly known as IVS1+1G>A), shown to impair splicing [12], has been identified in several cases, being particularly frequent in Czech Republic, Turkey, and Hungary [13–15].

A few studies have investigated, in addition to exon 1, the noncoding region immediately upstream of this exon, including the basal promoter [14, 16–21].

Houseman and coworkers [16] analysed HL patients heterozygous for c.101T>C (p.Met34Thr), in which no second *GJB2* coding mutation had been detected, and identified a monoallelic 10 bp deletion, c.-684_-675del (firstly designated -493del10), upstream of the basal promoter. The deletion was also present in other hearing impaired individuals as well as in control individuals, with or without c.101T>C. However, c.-684 -675del homozygosity was only observed in c.101T>C homozygous patients. The fact that in the control population 22 of the 25 (88%) c.101T>C heterozygotes carried the deletion suggested the existence of LD between c.101T>C and c.-684 -675del, later demonstrated by Zoll and coworkers [22]. Transcription was observed from alleles harbouring in *cis* the deletion and the variant c.101T>C, derived from keratinocytes and cell lines. However, eventual subtle differences would not have been detected, since this was not a quantitative analysis [16]. To date, the role of c.-684_-675del in HL has remained uncertain.

More recently, a pathogenic basal promoter mutation, c.-259C>T (firstly designated -3438C>T) was identified, in *trans* with c.250G>A (p.Val84Met), in a Portuguese HL patient, highlighting the relevance of screening *GJB2* noncoding regions in nonelucidated cases [18].

In the present study, we have analysed the basal promoter and the flanking upstream region, as well as the exon 1 and the 3'UTR of the *GJB2* gene in 89 Portuguese HL patients. The same analysis was conducted on 91 normal hearing control individuals from the Portuguese population.

2. METHODS

2.1. Subjects

Eighty-nine Portuguese HL patients previously screened for mutations in the *GJB2* coding region and acceptor splice site (by SSCP and/or sequencing) and for the del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) *GJB6* deletions (using the methodology described in [5]) were enrolled in this study. Eight patients were heterozygous for a *GJB2* coding mutation: c.71G>A (p.Trp24X; *n* = 1), c.35delG (*n* = 3), c.109G>A (p.Val37Ile; *n* = 1), c.380G>A (p.Arg127His; *n* = 1), c.457G>A (p.Val153Ile; *n* = 2), and one patient was heterozygous for the c.-22-12C>T variant (apparently a polymorphism; dbSNP accession number rs9578260). No patient harboured either of the known *GJB6* deletions. The HL was nonsyndromic in all patients, except for one of them, who presented with Waardenburg syndrome. The patient was heterozygous for the controversial c.457G>A mutation and was thus included in the study. The patients presented with bilateral, mild to profound HL, and were either familial or sporadic cases. The familial cases predominantly showed a recessive pattern of inheritance. All patients were audiologically evaluated by pure tone audiometry.

The control sample was composed of 91 Portuguese individuals with apparent normal hearing. The status regarding c.101T>C *GJB2* variant of those control individuals harbouring the c.-684_-675del, here referred, had been previously investigated, by sequencing, as part of an unpublished work. The status of the entire *GJB2* coding region is not known for the vast

majority of the 91 control individuals, which were blindly included in this study (and not based on their eventually available *GJB2* coding region status).

Informed consent was obtained from all the participants.

2.2. Genetic Analysis

In all individuals, we have sequenced a region of about 0.7 kb immediately upstream of the exon 1 (which includes the basal promoter), the exon 1, and the whole 3'UTR. The region upstream of the exon 1, plus exon 1 and donor splice site, was amplified in a 1009 bp amplicon, using the pair of primers PF2 5'-CgTTCgTTCggATTggTgAg-3' and PR1 5'-CAgAAACgCCCgCTCCAgAA-3', as previously described [18]. The amplicons were sequenced using the primers PF2 and PF1 5'-ggCTCAAAggAACTAggAgATCg-3'. When necessary, primers PR1 and PR2 5'-ggAgACTgggAAAgTTACgg-3' were used for sequencing. The 3'UTR (plus the last 90 nucleotides and stop codon) was amplified in three overlapping fragments using the following three pairs of primers: 3UTRaF 5'-gCAgTgTCTggAATTTgCATC-3', 3UTRaR 5'-AggCACTggTAACTTTgTCC-3', 3UTRbF 5'-CACgTTAAAggTgAACATTgg-3', 3UTRbR 5'-CgACAgAAACTTCTCCCTC-3', 3UTRcF 5'-gTAgCCAgCATCggAAAgAAC-3', 3UTRcR 5'-ACTCTggCAACTTACCCATTg-3'. The 3'UTR PCR products were sequenced using the respective amplification forward primers.

2.3. DNA Sequence Variants and SNPs Description

Description of variants follows the HGVS recommendations, and is based on the *GJB2* reference sequences accessed through the following links:

(1) https://research.cchmc.org/LOVD/refseq/GJB2codingDNA.html;

(2) https://research.cchmc.org/LOVD/refseq/GJB2intron 01.html;

(3) https://research.cchmc.org/LOVD/refseq/GJB2upstream.html;

(4) https://research.cchmc.org/LOVD/refseq/GJB2downstream.html.

These sequences show 100% identity with the NM 004004.5 (link 1) and NG 008358.1 (links 2, 3, and 4) NCBI reference sequences.

Novel variants were submitted to dbSNP and the respective reference SNP (rs) accession numbers are provided within the text.

SNPs are referred to by the dbSNP reference SNP (rs) accession number whenever it was available, and by the HGVS recommended designation, relative to the forementioned reference sequences.

2.4. Genotyping and Statistical Analysis

We have genotyped all individuals for the c.-684_-675del deletion; three SNPs in the promoter (rs9550621 (c.-484T>C), rs73431557 (c.-410T>C), rs9552101 (c.-369A>G)); ten SNPs in the 3'UTR (c.*1C>T, rs3751385 (c.*84T>C), rs7337074 (c.*104A>T), rs7329857 (c.*111C>T), rs55704559 (c.*168A>G), rs5030700 (c.*931C>T), rs1050960 (c.*1067G>T), rs7623 (c.*1152G>A), rs11841182 (c.*1197T>A), and rs7988691 (c.*1277T>C)); one SNP downstream of the 3'UTR (rs11839674 (c.*1447G>A)).

For the sake of simplicity, when describing the composite genotypes regarding SNPs rs73431557 (c.-410T>C), rs3751385 (c.*84T>C), rs55704559 (c.*168A>G), and rs5030700 (c.*931C>T), the genotype at each position, indicated in order from 5' to 3', is designated by A, C, G, or T if homozygous, or by a code letter, according to IUPAC nucleotide ambiguity code, if heterozygous.

The allelic frequencies regarding deletion c.-684_-675del and the 14 SNPs, were determined in the control population and used to test for Hardy-Weinberg equilibrium. The chi-square test was used to compare the allelic frequencies of the patients with those of the normal hearing individuals. Allelic frequencies of the control sample for the 14 SNPs were used to calculate pairwise linkage disequilibrium values. Testing for Hardy-Weinberg equilibrium, calculation of pairwise linkage disequilibrium values, and haplotype estimation (through the expectation maximization algorithm), were performed using SNPAnalyzer 1.2A online software (http://snp.istech.info/snp/SNPAnalyzer.html).

2.5. Analysis of mRNA Folding

Mfold (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form) [23] was used to assess the effect of alleles c.[*168A;*931C], c.[*168G;*931T], c.[*168A;*931T], and c.[*168G;*931C] on the folding of *GJB2* mRNA (template sequence: ENST00000382848, retrieved from Ensembl). For each sequence the lowest free-energy structure was considered.

3. RESULTS AND DISCUSSION

In the current study, 89 Portuguese HL patients, previously screened for mutations in the *GJB2* coding region and acceptor splice site (80 patients presenting no mutation, plus eight heterozygous for coding mutations and one heterozygous for the noncoding variant c.-22-12C>T), and 91 hearing individuals were analyzed as regards the noncoding region immediately upstream of the exon 1 (which includes the basal promoter), the exon 1, and the whole 3_UTR of *GJB2* gene. All individuals were also genotyped for c.-684_-675del and 14 SNPs localized therein.

3.1. DNA Sequence Variants

No additional *GJB2* variant was found in any of the eight patients previously found to be heterozygous for a coding *GJB2* mutation or in the patient heterozygous for the c.-22-12C>T noncoding variant.

Among the remaining 80 patients, six of them presented noncoding variants, which had already been reported (Table 1).

One patient, presenting with profound HL was heterozygous for the donor splice site c.-23+1G>A mutation. The patient may just be a carrier, or other *GJB2* or *GJB6* mutation might remain undetected. One other patient, presenting with moderate to severe HL, harboured in heterozygosity the c.-216T>G variant, located within the basal promoter, between two GT boxes [24, 25]. This variant was previously identified in two HL patients, also in heterozygosity [26]. The c.-45C>A variant in exon 1 was found in heterozygosity in one individual with severe HL.

This variant was referred, by Wilch and coworkers [8], as an SNP at position +94 in exon 1. These authors observed expression of the *GJB2* allele harbouring the variant but, since a quantitative comparison with wild-type allele was not performed, a possible contribution to HL cannot be excluded. Three affected individuals (two heterozygous and one homozygous) harboured the deletion c.-684_-675del.

No novel putative pathogenic noncoding mutation has been found in the patients, which might be due to the low number of monoallelic individuals and the small sample size. It is also possible that, simply, such mutations are very rare in our population.

Among controls, four novel noncoding variants were identified: c.-731C>T, c.-26G>T, c.*45G>A, and c.*985A>T (rs112400198, rs112875543, rs112399473, and rs111729919, respectively). Each of these variants was identified only once, in heterozygosity, and in different individuals (Table 1). The hearing individual harbouring the novel c.-731C>T variant was also heterozygous for the recessive c.670A>C (p.Lys224Gln) mutation (https://research.cchmc.org/LOVD/; phase unknown). One control individual harboured the c.-45C>A exon 1 variant in heterozygosity (Table 1). Interestingly, we found one control subject homozygous for c.-684 -675del (Table 1), which is, to our knowledge, the first case described to date of a normal hearing individual presenting this genotype. This individual did not harbour the c.101T>C mutation. Our finding, together with the previous report of transcription from alleles harbouring c.-684 -675del [16] suggests the nonpathogenicity of the deletion. In addition, six normal hearing heterozygotes for the deletion were also identified (Table 1), with one also heterozygous for c.101T>C.

It should be noted that the pathogenic basal promoter mutation c.-259C>T, identified for the first time in a Portuguese family [18], was not found among the 89 patients and 91 normal hearing individuals here analysed, and neither was it identified in the other studies which analysed the basal promoter [14, 16–21]. Therefore, known occurrence of c.-259C>T continues to be restricted to that Portuguese family.

3.2. Genotypic Data and Statistical Analysis

The allelic frequencies and Hardy-Weinberg equilibrium status regarding the deletion c.-684_-675del and the 14 noncoding SNPs were determined (Table 2; see Supplementary Table 1 in Supplementary Material available online at doi:10.4061/2011/827469).

The allelic frequencies of the deletion c.-684_-675del in patients and controls are not statistically different (Table 2). The allelic frequency observed for this deletion in our control population is close to the one found among the British control population [16], and higher than the one determined in the German control population [22].

The allelic frequencies regarding SNPs c.-410T>C, c.∗84T>C, c.∗168A>G, and c.∗931C>T, were statistically different between patient and control groups (Table 2).

By sorting both patients and controls into groups reflecting the genotypes for these four SNPs altogether, eleven composite genotypes were evidenced (Figure 1). Comparison of the genotypic frequencies in controls and patients promptly revealed an increased frequency in patients of the genotypes YYRY and CTRY, both heterozygous for SNPs c.∗168A>G and c.∗931C>T. Also, the genotype YCAC was identified in four patients but not found in controls. On the contrary, a decrease was observed in the frequency of the three genotypes that are most represented in controls-TCAC, TYAC, and YYAC. Each of the remaining genotypes was scarcely represented in both controls and patients (0%-2%), and their frequency did not varymore than 2%between the two groups; only 3% of controls and 4% of patients belong to one of these genotypes.

We also observed that, regarding SNPs c.∗168A>G and c.∗931C>T, nearly all individuals analysed (178/180) were either c.[=;=]+[=;=] or c.[∗168A>G(+)∗931C>T], which results from LD between these two SNPs (Supplementary Table 2, SNP pair 8 : 9). Interestingly, the overrepresentation of c.[∗168A>G(+)∗931C>T] genotype among patients, when comparing to hearing controls, is statistically very significant ($\chi 2$ = 28.159; P = 3.4 E-06), thus accounting for the statistically significant differences in the allelic frequencies of these two SNPs between patients and hearing controls.

The statistically significant differences also observed in the allelic frequencies of SNPs c.-410T>C and c.∗84T>C seems to be due to the differential association of their variants with the

estimated predominant alleles c.[*168A+*931C] and c.[*168G+*931T] (Tables 3(a) and 3(b)). This fact is in accordance with the observed LD between the two SNPs and SNPs c.*168A>G and c.*931C>T (Supplementary Table 2, SNP pairs 2 : 8, 2 : 9, 5 : 8, and 5 : 9).

It should be noticed that the presence of genotype YCAC among patients lends some contribute to the difference in allelic frequencies between patients and controls regarding SNP c.-410T>C.

The fact that YCAC genotype is not represented in 91 control individuals while it occurs in 4/89 patients is noteworthy. The presence of genotype YCAC implies the presence of haplotype CCAC, which frequency is of at least 2,2% among patients, and estimated to be null in the control population (as inferred from Table 3(a)). In order to validate a possible association of haplotype CCAC with HL analysis of larger samples of patients and normal hearing individuals is necessary. Interestingly, one of the four patients with the referred composite genotype is a c.457G>A heterozygote (phase unknown).

3.3. 3'UTR Variants and mRNA Folding

Our findings suggest that the c.[*168G;*931T] allele might have a deleterious effect, contributing to HL. We have used Mfold [23] to predict the effect of alleles c.[*168A;*931C], c.[*168G;*931T], c.[*168A;*931T], and c.[*168G;*931C] on mRNA folding. The change c.*168A>G, regardless of genotype at position c.*931, was predicted to alter mRNA folding. On the contrary, the change c.*931C>T, regardless of genotype at position c.*168, is not predicted to alter mRNA folding (Figure 2).

The c.*168A was predicted to be located in an internal loop of a stem-loop structure (Figure 2). Regulatory motifs in mRNA 3'UTR seem to function in the context of specific secondary structure [27]. Stem-loop structures occurring in the 3'UTR have been implicated in gene expression, with roles at the level of mRNA stability (e.g., the SLDE of G-CSF gene [28], the CDE of TNF-alpha gene [27, 29], the complex structure integrating three C-rich elements of alpha-globin gene, the histone mRNA 3' terminal stemloops, and the IRE of TFRC gene [27]) or translation (e.g., the common 30–37 nucleotide long element present in the target mRNAs of TIA-1, a translational repressor [30], and the SECIS element [27]). The disruption of the

predicted stem-loop structure and/or other adjacent stem-loop structures (Figure 2), induced by the c.*168A>G change, might lead to deregulation of the *GJB2* gene expression, thus being a contributor to the hearing loss phenotype. It should be stressed that mRNA folding predictions are fallible. This fact notwithstanding, the simple change of sequence, without affecting the secondary structure, could conceivably disrupt a binding site for a *trans*-acting factor, also leading to gene expression deregulation. Regarding the c.*931C>T variant, despite the predictions that c.*931C occurs in a helix and that the change from C to T does not have structural implications, the *in vivo* situation might be different. Functional studies involving constructs containing a reporter gene's coding sequence fused with *GJB2* 3'UTR could help elucidating the functional significance of these two sequence variants.

In this study, of a total of 15 patients presenting either a *GJB2* coding mutation or a noncoding variant, 14 do not harbour either the c.*168A>G or the c.*931C>T changes, whereas one patient, heterozygous for the controversial c.380G>A mutation, is a compound heterozygote regarding SNPs c.*168A>G and c.*931C>T (phase unknown). Therefore, our data do not allow withdrawal of conclusions concerning a putative role of the two 3_UTR variants in the HL of some monoallelic patients. In this regard, the investigation of the genotypes regarding c.*168A>G and c.931C>T variants in larger samples of monoallelic patients would be interesting. Finally, the finding of one c.*168G homozygote (a c.*931C>T heterozygote, and carrying no *GJB2* sequence variant) in our patient cohort, might further support a possible role of c.*168G in HL.

4. CONCLUSION

This study suggests the association of the noncoding SNPs c.*168A>G and c.*931C>T with HL. The c.*168A>G change is predicted to alter mRNA folding, suggesting a putative role of this SNP in the pathology.Our data also point to a possible association with HL of the haplotype CCAC, comprising SNPs c.-410T>C, c.*84T>C, c.*168A>G, and c.*931C>T, respectively. However, this observation requires validation through analysis of a larger number of subjects. The

technique of targeted sequence capture and massively parallel sequencing makes it very easy and cost-effective to screen large numbers of genes, and might cover noncoding sequences of some of them, such as *GJB2*. This approach could prove to be very useful for genetic diagnosis in cases of NSHL [31], with predictable benefits for genetic counseling of the affected families.

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REFERENCES

1. P. D. Stenson, M. Mort, E. V. Ball *et al*., "The human gene mutation database: 2008 update," *Genome Medicine*, vol. 1, no. 1, article gm13, 2009.

2. I. Lerer, M. Sagi, Z. Ben-Neriah, T. Wang, H. Levi, and D. Abeliovich, "A deletion mutation in GJB6 cooperating with a GJB2 mutation in trans in non-syndromic deafness: a novel founder mutation in Ashkenazi Jews," *Human Mutation*, vol. 18, no. 5, article 460, 2001.

3. I. del Castillo, M. Villamar, M. A. Moreno-Pelayo *et al*., "A deletion involving the connexin 30 gene in nonsyndromic hearing impairment," *The New England Journal of Medicine*, vol. 346, no. 4, pp. 243–249, 2002.

4. N. Pallares-Ruiz, P. Blanchet, M. Mondain, M. Claustres, and A. F. Roux, "A large deletion including most of GJB6 in recessive non syndromic deafness: a digenic effect?" *European Journal of Human Genetics*, vol. 10, no. 1, pp. 72–76, 2002.

5. F. J. del Castillo, M. Rodríguez-Ballesteros, A. Álvarez *et al*., "A novel deletion involving the connexin-30 gene, del(GJB6-d13s1854), found in trans with mutations in the GJB2 gene (connexin-26) in subjects with DFNB1 non-syndromic hearing impairment," *Journal of Medical Genetics*, vol. 42, no. 7, pp. 588–594, 2005.

6. J. E. A. Common, M. Bitner-Glindzicz, E. A. O'Toole *et al*., "Specific loss of connexin 26 expression in ductal sweat gland epithelium associated with the deletion mutation del(GJB6-D13S1830)," *Clinical and Experimental Dermatology*, vol. 30, no. 6, pp. 688–693, 2005.

7. J. Rodriguez-Paris and I. Schrijver, "The digenic hypothesis unraveled: the GJB6 del(GJB6-D13S1830) mutation causes allele-specific loss of GJB2 expression in cis," *Biochemical and Biophysical Research Communications*, vol. 389, no. 2, pp. 354–359, 2009.

8. E. Wilch, M. Zhu, K. B. Burkhart *et al*., "Expression of GJB2 and GJB6 is reduced in a novel DFNB1 allele," *American Journal of Human Genetics*, vol. 79, no. 1, pp. 174–179, 2006.

9. E. Wilch, H. Azaiez, R. A. Fisher *et al*., "A novel DFNB1 deletion allele supports the existence of a distant cis-regulatory region that controls GJB2 and GJB6 expression," *Clinical Genetics*, vol. 78, no. 3, pp. 267–274, 2010.

10. R. S. Mani, A. Ganapathy, R. Jalvi *et al*., "Functional consequences of novel connexin 26 mutations associated with hereditary hearing loss," *European Journal of Human Genetics*, vol. 17, no. 4, pp. 502–509, 2009.

11. F. Denoyelle, S. Mariin, D. Weil, L. Moatti, and P. Chauvin, "Clinical features of the prevalent form of childhood deafness, DFNB1, due to a connexin-26 gene defect: implications for genetic counselling," *The Lancet*, vol. 353, no. 9161, pp. 1298–1303, 1999.

12. H. Shahin, T. Walsh, T. Sobe *et al*., "Genetics of congenital deafness in the Palestinian population: multiple connexin 26 alleles with shared origins in the Middle East," *Human Genetics*, vol. 110, no. 3, pp. 284–289, 2002.

13. P. Seeman and I. Sakmaryová, "High prevalence of the IVS 1 + 1 G to A/GJB2 mutation among Czech hearing impaired patients with monoallelic mutation in the coding region of GJB2," *Clinical Genetics*, vol. 69, no. 5, pp. 410–413, 2006.

14. A. Sirmaci, D. Akcayoz-Duman, and M. Tekin, "The c.IVS1+1G>A mutation inthe GJB2 gene is prevalent and large deletions involving the GJB6 gene are not present in the Turkish population," *Journal of Genetics*, vol. 85, no. 3, pp. 213–216, 2006.

15. T. Tóth, S. Kupka, B. Haack *et al.*, "Coincidence of mutations in different connexin genes in Hungarian patients," *International Journal of Molecular Medicine*, vol. 20, no. 3, pp. 315–321, 2007.

16. M. J. Houseman, L. A. Ellis, A. Pagnamenta *et al.*, "Genetic analysis of the connexin-26 M34T variant: identification of genotype M34T/M34T segregating with mild-moderate non-syndromic sensorineural hearing loss," *Journal of Medical Genetics*, vol. 38, no. 1, pp. 20–25, 2001.

17. M. H. Chaleshtori, D. D. Farhud, R. Taylor, *et al.*, "Deafness—associated connexin 26 gene (GJB2) mutations in Iranian population," *Iranian Journal of Public Health*, vol. 31, no. 3, pp. 75–79, 2002.

18. T. D. Matos, H. Caria, H. Simões-Teixeira *et al.*, "A novel hearing loss-related mutation occurring in the GJB2 basal promoter," *Journal of Medical Genetics*, vol. 44, no. 11, pp. 721–725, 2007.

19. A. Pollak, M. Mueller-Malesińska, A. Skórka *et al.*, "GJB2 and hearing impairment: promoter defects do not explain the excess of monoallelic mutations," *Journal of Medical Genetics*, vol. 45, no. 9, pp. 607–608, 2008.

20. Y. Yuan, Y. You, D. Huang *et al.*, "Comprehensive molecular etiology analysis of nonsyndromic hearing impairment from typical areas in China.," *Journal of Translational Medicine*, vol. 7, article 79, 2009.

21. Y. Yuan, F. Yu, G. Wang *et al.*, "Prevalence of the GJB2 IVS1+1G >A mutation in Chinese hearing loss patients with monoallelic pathogenic mutation in the coding region of GJB2," *Journal of Translational Medicine*, vol. 8, article 127, 2010.

22. B. Zoll, L. Petersen, K. Lange *et al.*, "Evaluation of Cx26/GJB2 in German hearing impaired persons: mutation spectrum and detection of disequilibrium between M34T (c.101T>C) and -493del10," *Human Mutation*, vol. 21, no. 1, article 98, 2003.

23. M. Zuker, "Mfold web server for nucleic acid folding and hybridization prediction," *Nucleic Acids Research*, vol. 31, no. 13, pp. 3406–3415, 2003.

24. D. T. Kiang, N. Jin, Z. J. Tu, and H. H. Lin, "Upstream genomic sequence of the human connexin 26 gene," *Gene*, vol. 199, no. 1-2, pp. 165–171, 1997.

25. Z. J. Tu and D. T. Kiang, "Mapping and characterization of the basal promoter of the human connexin26 gene," *Biochimica et Biophysica Acta*, vol. 1443, no. 1-2, pp. 169–181, 1998.

26. H. Y. Tang, P. Fang, P. A. Ward *et al.*, "DNA sequence analysis of GJB2, encoding connexin 26: observations from a population of hearing impaired cases and variable carrier rates, complex genotypes, and ethnic stratification of alleles among controls," *American Journal of Medical Genetics, Part A*, vol. 140, no. 22, pp. 2401–2415, 2006.

27. J. M. Chen, C. Férec, and D. N. Cooper, "A systematic analysis of disease-associated variants in the 3′ regulatory regions of human protein-coding genes II: the importance of mRNA secondary structure in assessing the functionality of 3′ UTR variants," *Human Genetics*, vol. 120, no. 3, pp. 301–333, 2006.

28. C. Y. Brown, C. A. Lagnado, and G. J. Goodall, “A cytokine mRNA-destabilizing element that is structurally and functionally distinct from A+U-rich elements,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 24, pp. 13721–13725, 1996.

29. G. Stoecklin, M. Lu, B. Rattenbacher, and C. Moroni, “A constitutive decay element promotes tumor necrosis factor alpha mRNA degradation via an AU-rich element-independent pathway,” *Molecular and Cellular Biology*, vol. 23, no. 10, pp. 3506–3515, 2003.

30. I. López De Silanes, S. Galbán, J. L. Martindale *et al*., “Identification and functional outcome of mRNAs associated with RNA-binding protein TIA-1,” *Molecular and Cellular Biology*, vol. 25, no. 21, pp. 9520–9531, 2005.

31. A. E. Shearer, A. P. DeLuca, M. S. Hildebrand *et al*., “Comprehensive genetic testing for hereditary hearing loss using massively parallel sequencing,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 49, pp. 21104–21109, 2010.

FIGURES

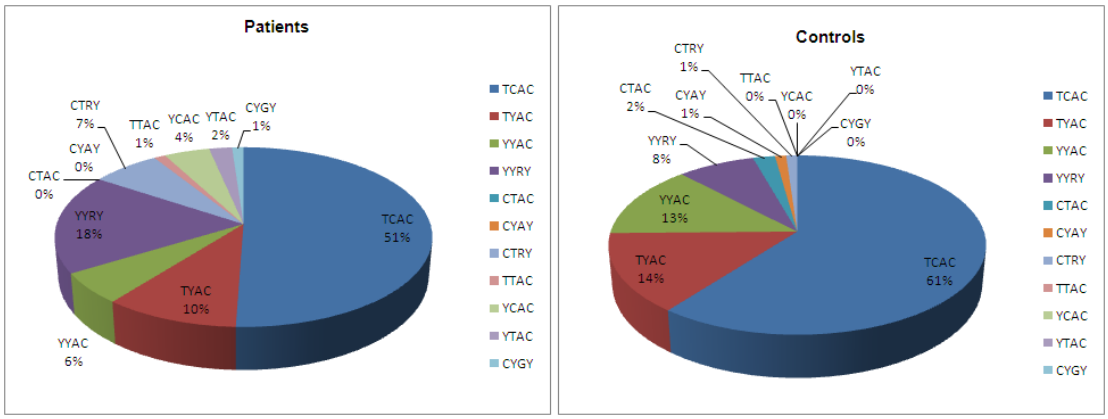


Figure 1. Frequencies, in patients (n=89) and controls (n=91), of the composite genotypes concerning SNPs c.-410T>C, c.*84T>C, c.*168A>G and c.*931C>T; R = A/G heterozygosity; Y = C/T heterozygosity (based on IUPAC nucleotide ambiguity code).

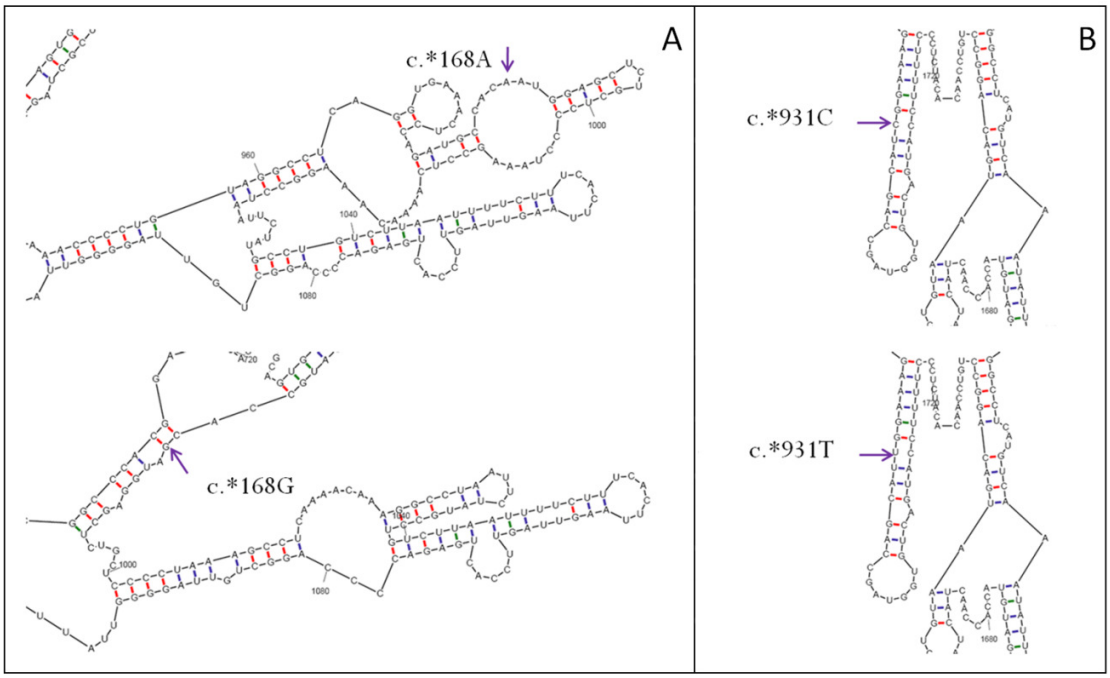


Figure 2. Effect of c.*168A>G and c.*931C>T changes in the 3'UTR on *GJB2* mRNA folding. A: c.*168A and c.*168G; B: c.*931C and c.*931T. The mRNA folding associated with each variant is the same regardless of the allele present at the other position, therefore only one example for each variant is provided.

TABLES

Table 1. *GJB2* variants identified in this study. Novel variants are in italic. BP- basal promoter; Ex 1 – exon 1; DSS – donor splice site; Ex 2 – exon 2; CR – coding region; 3' UTR – 3' unstranslated region.

Variant	Location	Patients (n=89)		Controls (n=91)	
		Heterozygote	Homozygote	Heterozygote	Homozygote
c.-731C>T	5' of the BP	0	0	**1**	0
c.-684_-675del	5' of the BP	**2**	**1**	**6**	**1**
c.-216T>G	BP	**1**	0	0	0
c.-45C>A	Ex 1	**1**	0	**1**	0
c.-26G>T	Ex 1	0	0	**1**	0
c.-23+1G>A	DSS	**1**	0	0	0
c.670A>C (p.Lys224Gln)	Ex 2 (CR)	0	0	**1**	0
c.*45G>A	3' UTR	0	0	**1**	0
c.*985A>T	3' UTR	0	0	**1**	0

Table 2. Differences in the allelic frequencies, regarding c.-684_-675del and 14 SNPs, between patient and control samples (chi-square test). ND – Not determined: chi-square test could not be performed for SNPs with an expected value <5, or for SNPs which both alleles were observed in only one sample. Four SNPs present statistically significant differences in allelic frequencies between patients and controls (P values <0.05, in bold).

Variant/SNP	Alleles	Patients (n=178 alleles)		Controls (n=182 alleles)	P value
		Observed	Expected		
c.-684_-675del	wt	174	170.18	174	0,162043
	c.-684_-675del	4	7.82	8	
rs9550621	c.-484 C	162	165.29	169	0,338941
	c.-484 T	16	12.71	13	
rs73431557	c.-410 C	41	26.41	27	**0,002089**
	c.-410 T	137	151.59	155	
rs9552101	c.-369 A	20	19.56	20	0,916103
	c.-369 G	158	158.44	162	
c.*1C>T	c.*1 C	177	178	182	ND
	c.*1 T	1	0	0	
rs3751385	c.*84 C	129	139.86	143	**0,04734**
	c.*84 T	49	38.14	39	
rs7337074	c.*104 A	174	177.02	181	ND
	c.*104 T	4	0.98	1	
rs7329857	c.*111 C	174	177.02	181	ND
	c.*111 T	4	0.98	1	
rs55704559	c.*168 A	154	170.18	174	**3,33E-09**
	c.*168 G	24	7.82	8	
rs5030700	c.*931 C	155	169.20	173	**9,18E-07**
	c.*931 T	23	8.80	9	
rs1050960	c.*1067 G	19	19.56	20	0,893155
	c.*1067 T	159	158.44	162	
rs7623	c.*1152 A	165	165.29	169	0,93373

	c.*1152 G	13	12.71	13	
rs11841182	c.*1197 A	2	0	0	ND
	c.*1197 T	176	178	182	
rs7988691	c.*1277 C	178	176.04	180	ND
	c.*1277 T	0	1.96	2	
rs11839674	c.*1447 A	2	0	0	ND
	c.*1447 G	176	178	182	

Table 3a. Estimated haplotype frequencies, based on the control population, concerning SNPs c.-410 T>C, c.*84T>C, c.*168A>G and c.*931C>T.

Haplotype	Frequency
TCAC	0.7802
CTAC	0.0989
TTAC	0.0714
CTGT	0.0440
CCAT	0.0055

Table 3b. Relationship between the estimated prevalent alleles regarding positions c.*168 and c.*931, and SNPs c.-410T>C and c.*84T>C.

Allele [c.*168; c.*931]	c.-410 T>C	c.*84T>C
AC	89.6% T	82.1% C
GT	100% C	100% T

SUPPLEMENTARY TABLES

Supplementary table 1. Analysis of Hardy-Weinberg equilibrium for c.-684_-675del and 14 SNPs. Calculations were based on the control population. ND – Not determined due to lack of genetic variability regarding the concerned SNP.

Variant/SNP	P value
c.-684_-675del	0.02972810
c.-484 T>C	0.39134875
c.-410 T>C	0.08837465
c.-369 A>G	0.32767174
c.*1C>T	ND
c.*84T>C	0.45797172
c.*104A>T	0.95828080
c.*111C>T	0.95828080
c.*168A>G	0.65802073
c.*931C>T	0.61631805
c.*1067G>T	0.91543627
c.*1152G>A	0.45797190
c.*1197T>A	ND
c.*1277T>C	0.91543627
c.*1447G>A	ND

Supplementary table 2. Analysis of pair-wise linkage disequilibrium regarding the 14 SNPs studied. Calculations were based on the control population. The P value and the scores for five measures of linkage disequilibrium (D, D', Delta, Delta squared and Fisher's) are presented for each pair of SNPs. Pair-wise linkage disequilibrium involving SNPs 4, 12 and 14 could not be determined due to lack of genetic variability regarding these SNPs. Correspondence: 1 - c.-484 T>C; 2 - c.-410 T>C; 3 - c.-369 A>G; 4 – c.*1C>T; 5 - c.*84T>C; 6 - c.*104A>T; 7 - c.*111C>T; 8 - c.*168A>G; 9 - c.*931C>T; 10 - c.*1067G>T; 11 - c.*1152G>A; 12 - c.*1197T>A; 13 - c.*1277T>C; 14 - c.*1447G>A.

SNPs Pair	P value	D	D'	Delta (Δ)	Delta squared (Δ^2)	Fisher's
1:2	0.1099	-0.0106	-1.0000	-0.1158	0.0134	1.1146
1:3	0.0000	0.0636	1.0000	0.7894	0.6231	2.0000
1:5	0.0000	0.0495	0.8812	0.4680	0.2190	2.0000
1:6	0.0000	0.0051	1.0000	0.2680	0.0718	2.0000
1:7	0.0000	0.0051	1.0000	0.2680	0.0718	2.0000
1:8	0.4167	-0.0031	-1.0000	-0.0595	0.0035	1.5460
1:9	0.3875	-0.0035	-1.0000	-0.0633	0.0040	1.5053
1:10	0.0000	0.0578	0.9094	0.7179	0.5153	2.0000
1:11	0.0000	0.0608	0.9167	0.9167	0.8403	2.0000
1:13	0.7716	-0.0004	-1.0000	-0.0207	0.0004	1.8619
2:3	0.0390	-0.0163	-1.0000	-0.1466	0.0215	1.0331
2:5	0.0000	0.1107	0.9495	0.7588	0.5758	2.0000
2:6	0.6723	-0.0008	-1.0000	-0.0310	0.0010	1.8516
2:7	0.6723	-0.0008	-1.0000	-0.0310	0.0010	1.8516
2:8	0.0000	0.0374	1.0000	0.5138	0.2639	2.000
2:9	0.0000	0.0421	1.0000	0.5465	0.2987	2.0000
2:10	0.0715	0.0144	0.1540	0.1297	0.0168	1.9467
2:11	0.1099	-0.0106	-1.0000	-0.1158	0.0134	1.1146
2:13	0.5484	-0.0016	-1.0000	-0.0440	0.0019	1.7246
3:5	0.0000	0.0470	0.5440	0.3660	0.1340	2.0000
3:6	0.0000	0.0049	1.0000	0.2115	0.0448	2.0000
3:7	0.0000	0.0049	1.0000	0.2115	0.0448	2.0000
3:8	0.3029	-0.0048	-1.0000	-0.0753	0.0057	1.3864
3:9	0.2726	-0.0054	-1.0000	-0.0801	0.0064	1.3420

3:10	0.0000	0.0518	0.5294	0.5294	0.2802	2.0000
3:11	0.0000	0.0578	0.9098	0.7182	0.5158	2.0000
3:13	0.7057	-0.0007	-1.0000	-0.0273	0.0007	1.7918
5:6	0.4675	0.0016	0.3671	0.0529	0.0028	2.0000
5:7	0.4675	0.0016	0.3671	0.0529	0.0028	2.0000
5:8	0.0000	0.0345	1.0000	0.4106	0.1686	2.0000
5:9	0.0000	0.0389	1.0000	0.4368	0.1908	2.0000
5:10	0.0000	0.0863	1.0000	0.6728	0.4527	2.0000
5:11	0.0000	0.0561	1.0000	0.5311	0.2821	2.0000
5:13	0.0000	0.0086	1.0000	0.2018	0.0407	2.0000
6:7	0.0000	0.0055	1.0000	1.0000	1.0000	2.0000
6:8	0.8281	-0.0002	-1.0000	-0.0159	0.0003	1.9560
6:9	0.8173	-0.0003	-1.0000	-0.0170	0.0003	1.9505
6:10	0.1877	0.0022	0.4414	0.0958	0.0092	2.0000
6:11	0.0715	0.0024	0.4645	0.1296	0.0168	2.0000
6:13	0.9150	-0.0001	-1.0000	-0.0078	0.0001	1.9890
7:8	0.8281	-0.0002	-1.0000	-0.0159	0.0003	1.9560
7:9	0.8173	-0.0003	-1.0000	-0.0170	0.0003	1.9505
7:10	0.1877	0.0022	0.4414	0.0958	0.0092	2.0000
7:11	0.0715	0.0024	0.4645	0.1296	0.0168	2.0000
7:13	0.9150	-0.0001	-1.0000	-0.0078	0.0001	1.9890
8:9	0.0000	0.0418	1.0000	0.9401	0.8838	2.0000
8:10	0.8877	0.0007	0.0170	0.0104	0.0001	1.7853
8:11	0.4167	-0.0031	-1.0000	-0.0595	0.0035	1.5460
8:13	0.7580	-0.0005	-1.0000	-0.0226	0.0005	1.9138
9:10	0.9903	0.0001	0.0014	0.0009	0.0000	1.7417
9:11	0.3875	-0.0035	-1.0000	-0.0633	0.0040	1.5053
9:13	0.7431	-0.0005	-1.0000	-0.0240	0.0006	1.9033
10:11	0.0000	0.0636	1.0000	0.7894	0.6231	2.0000
10:13	0.0000	0.0098	1.0000	0.3000	0.0900	2.0000
11:13	0.0000	0.0102	1.0000	0.3801	0.1444	2.0000

ANNEX

Figures

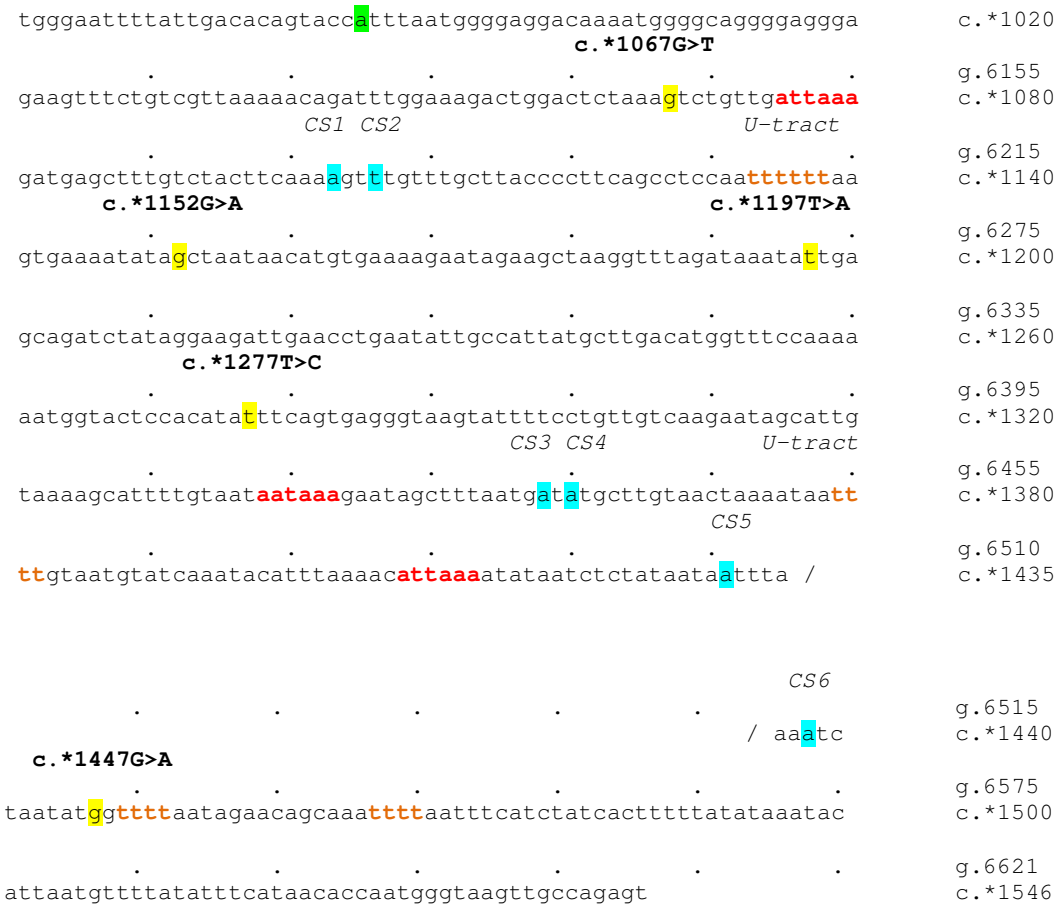
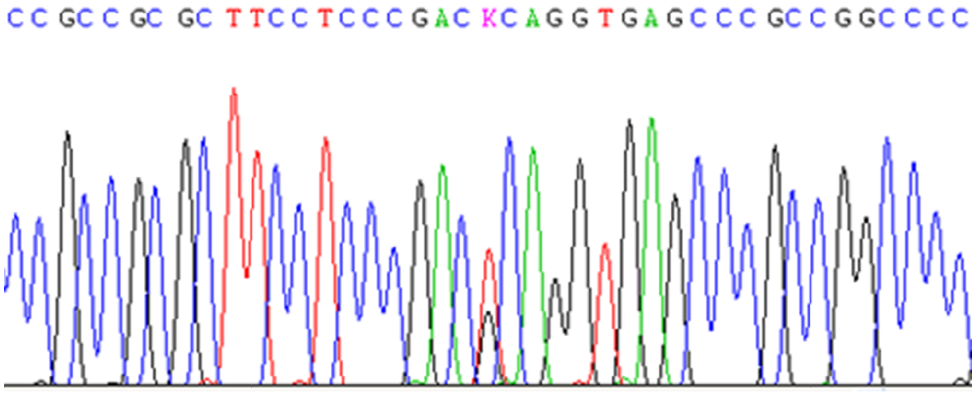


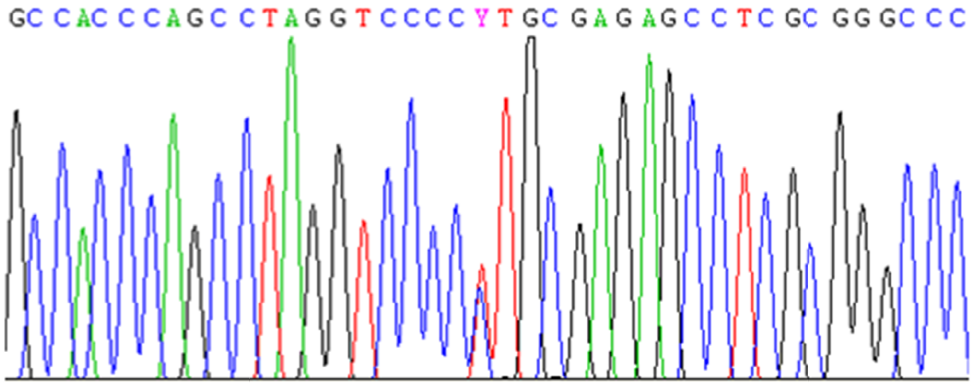
Figure 1. Human *GJB2* genomic sequence including the whole 3'UTR, and the further downstream sequence analysed in this work. The six inferred cleavage sites (CS1-6), highlighted in blue, were assigned based on the human sequences deposited at Genbank with the following accession numbers: CS1 - CA310936.1 (lung epithelial cells), AW086214.1 (ovarian carcinoma); CS2 - BC071703.1 (adult colon, kidney and stomach pool); CS3 - AA613715.1 (colon tumor); CS4 - AA461087.1 (total 8-9 weeks fetus); CS5 - BI492740.1 (fetal cochlea), BC017048.1 (colon adenocarcinoma), BU686041.1 (lung epithelial cells), CD370089.1 (lung alveolar macrophage); CS6 - BI491091.1 (fetal cochlea); corresponds to the NM_004004.5 mRNA reference sequence. CS1, CS4 and CS5 correspond to the three alternative polyadenylation sites in AceView (D. Thierry-Mieg & Thierry-Mieg, 2010), although the exact location of each CS seems to differ from those here presented. The counting criteria of the number of bases between the UPCAS and the poly-A site did not seem to be uniform but, apparently, AceView (D. Thierry-Mieg & Thierry-Mieg, 2010) considered the CS to be the last non-A residue before the poly-A tail even if it included genomic As. Here, in such cases, since it is possible that the first nucleotide of the poly-A tail might be template encoded (Zhao, Hyman, & Moore, 1999), we have considered a genomic A (arbitrarly

the last) to be the CS. The 11 investigated SNPs within the 3'UTR are highlighted in yellow, while the two novel SNPs, within the same region, identified in this work, are highlighted in green (the names of the SNPs are provided above their locations). The UPCAS are in red and bold font. The potential DCPAS (U-stretches in the corresponding pre-mRNA) are in orange and bold font. The presented sequence was accessed through **Public** CCHMC Molecular Genetics Laboratory Mutation Database - gap junction protein, beta 2, 26kDa (GJB2) (Husami *et al*, 2009a, 2009b).

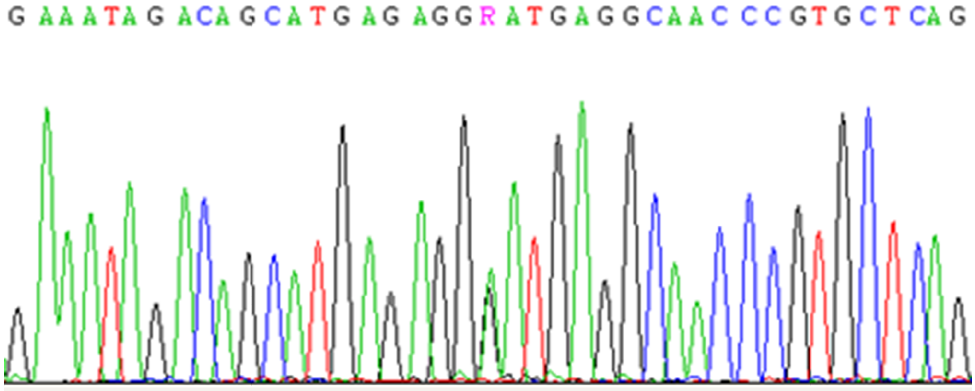
c.-26G>T



c.-731C>T



c.*45G>A



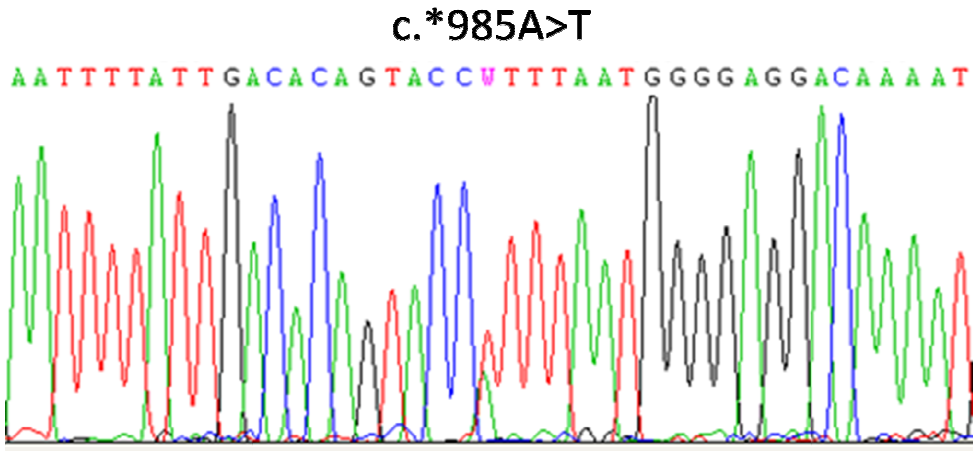


Figure 2. Partial DNA sequencing electrophoretograms showing the novel *GJB2* sequence variants identified in this study. The designation of each sequence variant is provided above the corresponding electrophoretogram.

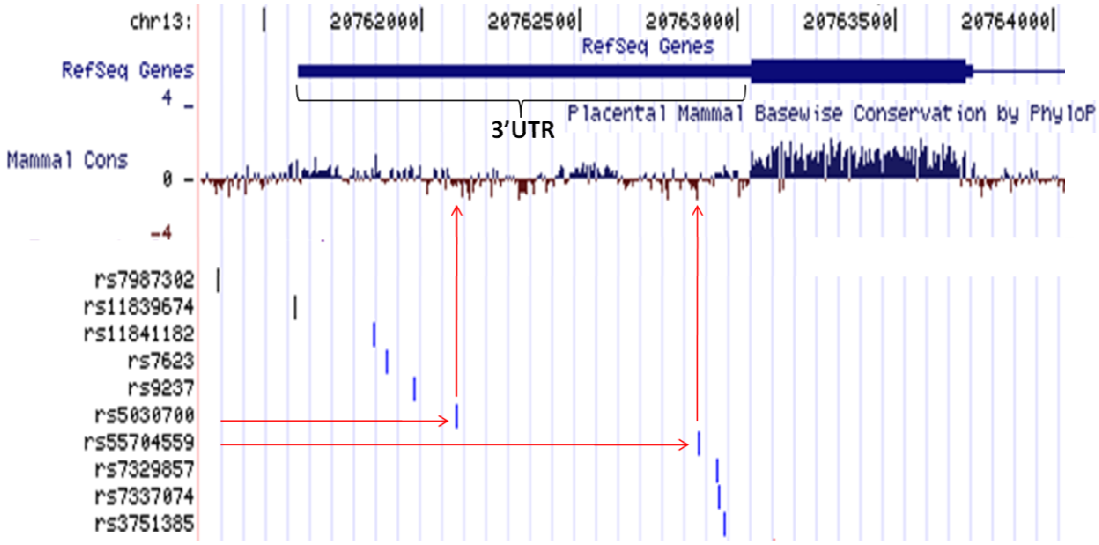


Figure 3. Conservation of the 3'UTR of the *GJB2* gene. Some regions within the 3'UTR show considerable degree of conservation, and do not include the SNPs c.*168A>G (rs55704559) and c.*931C>T (rs5030700). Adapted from Encode database (Birney *et al.*, 2007).

References

Birney, E., Stamatoyannopoulos, J. A., Dutta, A., Guigo, R., Gingeras, T. R., Margulies, E. H., Weng, Z. P., *et al*. (2007). Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature*, *447*(7146), 799-816.

Husami *et al*. (2009a). gap junction protein, beta 2, 26kDa (GJB2) - downstream reference sequence. ***Public** CCHMC Molecular Genetics Laboratory Mutation Database - gap junction protein, beta 2, 26kDa (GJB2)*. Retrieved December 7, 2011, a from https://research.cchmc.org/LOVD/refseq/GJB2_downstream.html

Husami *et al*. (2009b). gap junction protein, beta 2, 26kDa (GJB2) - coding DNA reference sequence. ***Public** CCHMC Molecular Genetics Laboratory Mutation Database - gap junction protein, beta 2, 26kDa (GJB2)*. Retrieved December 7, 2011, b from https://research.cchmc.org/LOVD/refseq/GJB2_codingDNA.html

Thierry-Mieg, D., & Thierry-Mieg, J. (2010). Homo sapiens gene GJB2, encoding gap junction protein, beta 2, 26kDa. *AceView: integrative annotation of cDNA-supported genes in human, mouse, rat, worm and Arabidopsis*. Retrieved April 13, 2011, from http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?db=human&term=GJB2

Zhao, J., Hyman, L., & Moore, C. (1999). Formation of mRNA 3' Ends in Eukaryotes: Mechanism, Regulation, and Interrelationships with Other Steps in mRNA Synthesis. *Microbiology and Molecular Biology Reviews*, *63*(2), 405-445.

CHAPTER 7

General Discussion

The initial part of the work leading to this PhD thesis consisted in searching for mutations in the coding region and acceptor splice site of the *GJB2* gene in over 140 Portuguese individuals, presenting with NSSHL and already screened for the c.35delG mutation. Since mutations in this single gene were shown to account for a significant fraction of the NSSHL cases in many populations (Kenneson, Van Naarden Braun, & Boyle, 2002), *GJB2* molecular analysis has been the first step of the studies on hereditary HL led by our group in Portugal since 1998.

During this past activity, two novel coding *GJB2* mutations, p.Val84Met and p.Met163Leu were identified (Matos, 2002; Matos, Caria, O'Neill, Correia, & Fialho, 2003). A short time after, the p.Val84Met mutation was also found by other group (Pandya *et al*., 2003). Additionally, the less frequently studied basal promoter, exon 1 and donor splice site were investigated in some patients, found to be heterozygous for a recessive, controversial or unclear mutation. As a result of this additional mutational screening, a previously unreported mutation (c.-259C>T) was found within the basal promoter, in *trans* with the p.Val84Met mutation (Matos *et al*., 2007).

The results obtained were integrated in the overall genetic data concerning the prevalence of *GJB2* mutations in 264 Portuguese sporadic and familial NSSHL cases (Chapter 2). The results pertaining three of the families analysed in this first stage of the study, all carrying the controversial p.Arg127His mutation, were discussed in more detail in Chapter 5.

The second part of this work consisted in the functional characterization of the fore mentioned c.-259C>T, p.Val84Leu and p.Met163Leu mutations, in order to elucidate their suspected pathogenicity and contribute for a better understanding of the effects of the different *GJB2* mutations and of how they conduce to HL (Chapters 3 and 4).

The final part of this work, presented in Chapter 6, included the extension of the pilot study concerning noncoding regions, by screening the *GJB2* donor splice site, exon 1 and the immediately 0.7 Kb upstream region (including the basal promoter), and the whole 3'UTR, in a larger sample of HL patients (n=89) who harboured none or just one *GJB2* coding mutation. Furthermore, a total of 14 SNPs across the fore mentioned noncoding regions were genotyped in these patients. The same methodology was followed to study 91 individuals with self-reported normal hearing from the Portuguese general population.

1. Spectrum of mutations in the *GJB2* gene in Portuguese NSSHL patients

We have found a wide spectrum of *GJB2* pathogenic/controversial mutations among the 264 Portuguese NSSHL families analysed (Chapter 2). Most of these mutations had already been identified in other populations. Three novel mutations (c.-259C>T, p.Val84Met and p.Met163Leu) were found in two of these Portuguese families (Matos, 2002; Matos *et al*., 2003, 2008, 2007). One of these three mutations (c.-259C>T) has, until now, not been reported in any family other than the one in which it was identified (Matos *et al*., 2007).

Taking into consideration the overall results regarding the 264 families, some important conclusions can be drawn.

GJB2 mutations, in coding and noncoding regions, were found in 80 (30.2%) of the 264 patients. Patients with two *GJB2* mutations represented 20% of the probands analysed, while 10.2% harboured only one *GJB2* variant.

The c.35delG deletion was, as expected, the *GJB2* mutation most frequently found amongst the NSSHL patients, comprising 63.2% of the mutant alleles, and being present in over 80% of the biallelic individuals. The c.35delG homozygous genotype was by far the most prevalent *GJB2* mutated genotype, being present in 10.6% of the Portuguese NSSHL patients. In accordance with other studies (Cryns *et al*., 2004; Murgia *et al*., 1999), we also noticed variability in the degree of HL in the patients homozygous for c.35delG.

The HL of the patients could be attributed, or assumed to be due, to the *GJB2* genotype in all biallelic patients, with the exception of the only p.Arg127His homozygous patient, since we could not determine whether this controversial genotype (Chapter 5) was segregating with the HL in the family.

The *GJB2* genotype of one monoallelic patient also explained the HL, since the patient harboured one dominant *GJB2* mutation (p.Met163Leu). Interestingly, the dominant *GJB2* mutation p.Met163Leu, causing mild/moderate high-frequency HL in the Portuguese patient and her mother (Chapter 4), has meanwhile also been found in one Argentinean patient presenting with prelingual, profound NSSHL (Dalamón *et al*., 2010), suggesting that the severity of the HL due to the p.Met163Leu mutation might be variable and/or progressive.

Further screening for the *GJB6* deletions, and *GJB2* basal promoter and donor splice site, contributed to the elucidation of the HL in five other patients with only one coding mutation, since the del(*GJB6*-D13S1854) was found in two of these patients, the basal

promoter (c.-259C>T) mutation was identified in another one, and the donor splice site (c.-23+1G>A) mutation was present in the remaining two individuals.

Interestingly, an excess of c.35delG heterozygotes (11/264) was observed. Their allelic frequency corresponds to 4.1%, which is very high when compared with the c.35delG carrier rate in the Portuguese general population (0.88%) (data not yet published). It should be noted that we were not able to analyse all the 11 patients for the *GJB6* deletions and the *GJB2* noncoding regions considered in this study. So, although some of these individuals might just be coincidental carriers, some other patients may harbour a second DFNB1 mutation, even in other *GJB2/GJB6* regions not here analysed, thus explaining the excess of c.35delG heterozygotes.

In conclusion, with basis solely on coding mutations, a diagnosis of *GJB2*-associated HL was confirmed for 18.9% of the cases. Thus, *GJB2* mutations are an important cause of NSSHL in the Portuguese population, being c.35delG the predominant mutation, with each of the 19 other pathogenic/controversial variants occurring at much lower frequencies. Screening for the *GJB2* donor splice site and the basal promoter, as well as for the two common *GJB6* deletions, allowed the elucidation of the etiology of the HL in some of the cases with only one coding mutation identified. Therefore, such screening, in monoallelic patients, is advisable and should be performed.

Overall, 20.8% of the patients were diagnosed as having DFNB1-related NSSHL, which represents a valuable indicator as regards therapeutical and rehabilitation options, as well as genetic counseling of Portuguese patients and their families.

1.1 Genetic influx from other populations

The identification, in Portuguese HL patients, of certain variants (c.-23+1G>A, c.-22-12C>T, p.Trp24X, p.Val37Ile, c.167delT and p.Arg127His) which are particularly frequent in certain populations, is suggestive of genetic influx.

p.Thr24X. The p.Trp24X mutation is a frequent variant among NSHL patients in India, where high carrier frequencies were observed, although the prevalence among normal-hearing control individuals seemed to vary with the geographic region (Joseph & Rasool, 2009; Khandelwal, Bhalla, Khullar, & Panda, 2009; Maheshwari *et al*., 2003; G. Padma, Ramchander, Nandur, & Padma, 2009; RamShankar *et al*., 2003). This mutation is also frequent among NSHL cases in Gypsies, an ethnic group tracing their origins to the Indian

subcontinent (Minárik *et al*., 2003; Álvarez *et al*., 2005), and presents with high carrier frequencies in several European Gypsy subpopulations (Bouwer *et al*., 2007). In line with these data, in our patient sample those harbouring the p.Trp24X mutation are of Indian or Gypsy descent.

p.Arg127His. The p.Arg127His mutation is a common variant in the Indian population (G. Padma *et al*., 2009; RamShankar *et al*., 2003), having also been identified in Gypsy NSHL patients (Minárik *et al*., 2003; Álvarez *et al*., 2005). In our study, this mutation was identified in five families, three of which are described in Chapter 5. Two of these five families belong to the Gypsy ethnicity, being the ethnicity of the other three families unknown.

p.Val37Ile. As regards p.Val37Ile, its prevalence is high in Eastern Asians (Abe, Usami, Shinkawa, Kelley, & Kimberling, 2000; Han *et al*., 2008; Huculak, Bruyere, Nelson, Kozak, & Langlois, 2006; Hwa *et al*., 2003; Kudo *et al*., 2000; Ohtsuka *et al*., 2003; Wattanasirichaigoon *et al*., 2004). Although this mutation presented a high allelic frequency (15.3%) among Asian patients, significant allelic frequencies were found among Hispanic (1.1%) and White (5.5%) hearing-impaired individuals in the study conducted by Putcha *et al*. (2007). Therefore, the presence of p.Val37Ile at considerable frequencies in HL patients, across distinct ethic groups, might reflect ancient migrations.

c.-23+1G>A. The c.-23+1G>A donor splice site mutation has been frequently identified in NSHL cases in Eastern Europe, Turkey and the Kurdish population of Iran (Mahdieh *et al*., 2004; Seeman & Sakmaryová, 2006; Sirmaci, Akcayoz-Duman, & Tekin, 2006; Tóth *et al*., 2007).

c.167delT. The carrier frequency of c.167delT is very significant in Ashkenazi Jews (Lerer *et al*., 2000; Morell *et al*., 1998; Sobe *et al*., 1999). This mutation was also found at relatively high frequency in Palestinian NSSHL patients, and is thought to have occurred a single time in Middle Eastern antiquity (Shahin *et al*., 2002). Given the presence of Jews in Portugal since many centuries ago, it is not surprising to find the c.167delT mutation in Portuguese patients.

c.-22-12C>T. The c.-22-12C>T variant (dbSNP accession number rs9578260) seems to be mostly associated with subsaharian Africans (Bethesda (MD): National Center for Biotechnology Information National Library of Medicine, n.d.). The finding of this variant among Portuguese patients is also not surprising due to the historic relation between Portugal and some subsaharian African countries (former Portuguese colonies).

1.2. Controversial mutations

The mutations p.Met34Thr, p.Arg127His, p.Val153Ile and p.Gly160Ser, identified in this study, are of controversial pathogenicity.

p.Met34Thr. Heterozygosity for p.Met34Thr occurring in normal-hearing individuals has been reported by Scott *et al*. (1998) and several other studies, including the present one (Chapter 2), while all p.Met34Thr homozygotes described so far showed HL, most often ranging from mild to moderate (R. A. Cucci *et al*., 2000; Dietz, Löppönen, Valtonen, Hyvärinen, & Löppönen, 2009; Houseman *et al*., 2001; Marlin *et al*., 2001; Snoeckx, Huygen, *et al*., 2005; Teek *et al*., 2010; B.-L. Wu *et al*., 2002). Altogether, these reports support the view of p.Met34Thr being a recessive mutation.

Skerrett, Di, Kasperek, Kelsell, and Nicholson (2004) presented electrophysiological evidence that also supports a recessive role for the p.Met34Thr allele. Impaired function of intercellular channels composed of p.Met34Thr-Cx26 was further supported by other studies, as assessed by single channel conductance measurements, intercellular neurobiotin (NBN) and LY transfer and Ca^{2+} waves spreading assays (Bicego *et al*., 2006; D'Andrea *et al*., 2002; Thönnissen *et al*., 2002).

Normal-hearing individuals who were compound heterozygous for p.Met34Thr and c.35delG or del(*GJB6*-D13S1830) have been described (Feldmann *et al*., 2004). Nonetheless, these and other compound heterozygous genotypes involving p.Met34Thr, have also been found in individuals with some degree of hearing impairment, most often presenting with mild to moderate HL (Bicego *et al*., 2006; Cama *et al*., 2009; Griffith *et al*., 2000; Kenna, Wu, Cotanche, Korf, & Rehm, 2001; Snoeckx, Huygen, *et al*., 2005; B.-L. Wu *et al*., 2002). Pollak *et al*. (2007) found a clear association of c.35delG/p.Met34Thr genotype with HL, but of relatively mild severity, and with weaker penetrance compared with c.35delG homozygotes. The authors found that the HL associated with c.35delG/p.Met34Thr genotype was often detected only at > 7 years old, and provided evidence supporting the progressiveness of the impairment. Thus, penetrance of DFNB1 compound genotypes involving the p.Met34Thr mutation might depend on genetic modifiers and/or environmental factors.

In two patients of this study we have found the p.Met34Thr mutation in compound heterozygosity with the c.35delG and p.Val95Met recessive mutations, respectively. Thus, both compound genotypes were assumed to be the likely causes of the HL.

p.Arg127His. Regarding the p.Arg127His variant, identified in this study in a total of five families, we have performed a detailed review of the literature aiming at clarifying its role. Many authors argue that evidence plays in favor of it being a polymorphism. However, considering our genetic data, as well as the genetic and functional data available in the literature, our opinion is that the p.Arg127His mutation might exert a pathogenic effect, depending on the genetic background and/or environmental factors (Chapter 5).

p.Val153Ile. The p.Val153Ile mutation, which we found as the sole *GJB2* mutation in Portuguese patients (Chapters 2 and 6), has been considered as either a polymorphism or a recessive allele. Marlin *et al*. (2001) refer two cases in which two individuals with normal hearing (at least at conversational frequencies) had the c.35delG/p.Val153Ile genotype, and RamShankar *et al*. (2003) refer two hearing individuals homozygous for p.Val153Ile as well as similar frequencies of p.Val153Ile in hearing-impaired and in hearing individuals. Nonetheless, several studies reported the identification, in HL patients, of the p.Val153Ile mutation in compound heterozygosity with the recessive *GJB2* mutations c.35delG, p.Thr8Met and p.Leu90Pro (Cryns *et al*., 2004; Dalamón *et al*., 2005; Hashemzadeh Chaleshtori *et al*., 2005; Kenna *et al*., 2001; Marlin *et al*., 2001; Snoeckx, Hassan, Kamal, Van Den Bogaert, & Van Camp, 2005), or with p.Met34Thr (Snoeckx, Huygen, *et al*., 2005). Noteworthy, the genotype p.Thr8Met/p.Val153Ile has been reported, in HL patients, in four different studies (in distinct genetic backgrounds). Moreover, the p.Val153Ile mutant protein was unable to form functional intercellular channels in *Xenopus* oocytes (Meşe, Londin, Mui, Brink, & White, 2004). However, a more recent study, this time in a mammalian expression system (HeLa cells), reported that channels composed of the p.Val153Ile mutant connexin were targeted to the membrane and formed intercellular channels permeable to LY (Guerci *et al*., 2007). It should be noted that normal permeability to LY has been reported for the pathogenic p.Val84Leu mutation, which displayed impaired permeability to IP3 (Beltramello, Piazza, Bukauskas, Pozzan, & Mammano, 2005). Considering all these data, a pathogenic effect of the p.Val153Ile mutation remains a possibility, and should not be disregarded.

p.Gly160Ser. Mutation p.Gly160Ser was identified in a proband (sporadic case) presenting with bilateral moderate NSSHL at the age of 11 years old (Chapters 2 and 3). This mutation seems to be a rare variant (<1/~150 alleles) in the general population across different ethnic groups (Tang *et al*., 2006), and has usually been regarded as a polymorphism. The p.Gly160Ser mutation, in heterozygosity, was found in two NSHL cases

compatible with dominant HL, reported by Janecke *et al*. (2002) and Löffler *et al*. (2001). However, the genetic data presented do not allow conclusions to be drawn regarding a role of p.Gly160Ser in HL. Some other HL patients, heterozygous for the p.Gly160Ser mutation, have been reported (Cheng *et al*., 2005; Hashemzadeh Chaleshtori, Farhud, & Patton, 2007; Santos *et al*., 2005; Tang *et al*., 2006) but, considering the presented information, no inference about its pathogenicity can be made. On the other hand, the report of hearing-impaired individuals presenting with NSHL compatible with a recessive mode of inheritance and with the c.35delG/p.Gly160Ser or p.Val37Ile/p.Gly160Ser genotypes (Snoeckx, Huygen, *et al*., 2005) could suggest a possible pathogenic effect of the p.Gly160Ser mutation. Further genetic data and/or functional studies are needed to help elucidating the role of p.Gly160Ser in HL.

2. Assessment of *GJB2* noncoding regions in HL patients

2. 1. Exon 1, basal promoter and upstream region

Most of the studies that analysed the *GJB2* gene of HL patients have not screened the gene beyond its coding region and splice sites. However, the whole exon 1 and the upstream region (including the basal promoter) have been analysed in some studies. In spite of the fact that these noncoding regions have not been given as much attention as the coding region, they are potential locations of pathogenic mutations which might interfere with *GJB2* expression. These mutations could help to elucidate and explain the excess of c.35delG monoallelic patients identified in the Portuguese patients analised.

We have investigated *GJB2* donor splice site, exon 1 and basal promoter in a total of 100 unrelated HL patients, previously analysed as regards the coding region and not harbouring any of the *GJB6* common deletions (Chapters 3, 4, 5 and 6). Eighteen out of these 100 patients carried one pathogenic or controversial *GJB2* coding mutation.

As a result of this screening, we have found, in one of the monoallelic patients, above reported, a novel *GJB2* mutation, c.-259C>T, occurring within a Sp1/Sp3 binding site in the *GJB2* basal promoter (Chapter 3). This mutation was identified in *trans* with the c.250G>A (p.Val84Met) mutation in a profoundly hearing-impaired individual. The patient's moderately hearing-impaired mother was heterozygous for the p.Val84Met mutation only,

while her normal-hearing sister was heterozygous for the basal promoter mutation. The location of the mutation in a transcription factor binding site critical for *GJB2* basal expression, its occurrence in *trans* with a coding *GJB2* mutation in an hearing-impaired individual, and its absence in 70 unrelated normal-hearing controls, were suggestive of a possible pathogenic effect. Therefore, we have performed a functional study in order to assess its functionality (Chapter 3).

In one other HL patient, carrier of the controversial p.Arg127His, already mentioned above, we have identified the c.-23+1G>A donor splice site recessive mutation (Chapter 5). The compound genotype was considered as a likely cause of the HL. The same noncoding mutation was identified in heterozygosity in one other patient, who didn't harbour any *GJB2* coding mutation (Chapter 6). The etiology of the HL in that case remained unelucidated.

One important finding of this study was the identification, for the first time, of a normal-hearing individual homozygous for the 10 bp deletion c.-684_-675del (Chapter 6) and not carrying the p.Met34Thr mutation, which is in LD with the 10 bp deletion (Zoll *et al*., 2003). Whether this deletion has a pathogenic effect on the hearing function has been unclear. Although it had been previously found in homozygosity in two hearing-impaired individuals, these two sibs were also homozygous for the p.Met34Thr mutation (Houseman *et al*., 2001). The same study reported expression from the allele harbouring both c.-684_-675del and p.Met34Thr mutations, but this analysis was not quantitative. The normal-hearing individual homozygous for the 10bp deletion, here reported, provides evidence for its non-pathogenicity. To our knowledge, after the first report of the c.-684_-675del by Houseman *et al*. (2001) only the study of Zoll *et al*. (2003) and our own have considered the c.-684_-675del. These three studies revealed considerable allelic frequencies in the British, German and Portuguese (this study) control populations (4.9%, 2.8% and 4.4%, respectively).

We have also identified, in normal-hearing subjects, two previously unreported variants in the exon 1 and in the region upstream of the basal promoter (see sequencing traces in Chapter 6, Annex fig. 2).

2.2. 3'UTR

As already mentioned, most of the studies on *GJB2* have only analysed the coding region. Some of these studies, however, have also analysed the sequence immediately downstream of the stop codon (included in the amplified region) but they have not

considered the whole 3'UTR. We could find in the literature one study in which the whole 3'UTR of the *GJB2* gene had also been analysed, but only in three patients from a large pedigree (Wilch *et al*., 2006). No putative pathogenic variant in the 3'UTR has been reported in that or in any other study.

The 3'UTRs play, however, important roles in gene expression, being involved in its regulation at multiple levels (mRNA 3'-end formation, mRNA polyadenylation, stability/degradation, nuclear export, subcellular localisation and translation efficiency), through a variety of *cis*-acting regulatory elements (reviewed in J.-M. Chen, Férec, & Cooper, 2006a, 2006b). Therefore, 3'UTRs should be considered in the genetic screening of disease-associated genes. In fact, several sequence variants (including common polymorphisms in the general population) within the 3'UTR of other genes have been shown to affect gene expression (for instance, by interfering with mRNA 3'-end formation or mRNA stability) leading to or being associated with human disease (reviewed in J.-M. Chen, Férec, & Cooper, 2006a, 2006b).

Thus, we have sequenced and analysed the whole *GJB2* 3'UTR in 89 unrelated HL patients (who are included in the 100 patients analysed for the *GJB2* donor splice site, exon 1 and basal promoter), who were negative or heterozygous for pathogenic or controversial *GJB2* coding mutations, as well as in 91 normal-hearing controls. This analysis also included the genotyping for ten SNPs within the 3'UTR, and one SNP further downstream, for which we observed variability in the patient and/or control samples.

Polyadenylation of eukaryotic mRNAs is critical for the regulation of mRNA stability and translation. In mammalian, three elements define the core polyadenylation signal: a) the UCPAS (upstream core polyadenylation signal), a conserved hexamer (AATAAA or a close variant), located 10-30 nucleotides upstream the polyadenylation addition site; b) the DCPAS (downstream core polyadenylation signal) which is a less conserved U/GU rich element, located 10-30 nucleotides downstream the polyadenylation addition site; c) the cleavage site (CS), or the polyadenylation addition site (J.-M. Chen, Férec, & Cooper, 2006a). The UPCAS is a potential location of pathogenic mutations. In fact, some mutations were shown to disrupt this signal in other genes and are associated with human disease (reviewed in Chatterjee & Pal, 2009; J.-M. Chen, Férec, & Cooper, 2006a). Furthermore, the identity of the nucleotide residues at positions -1 and -2 relative to the CS appear not to be random, since Sheets, Ogg, and Wickens (1990) observed that these positions were occupied by an adenine and cytosine

residues, respectively, in most of the vertebrate genes they have analysed. Consistent with the suggested functional relevance of the two positions immediately upstream of the CS, a polymorphism at position -1 relative to the CS of the prothrombin gene (F2) was found to be associated with elevated prothrombin levels and with increased risk of venous thrombosis (Poort, Rosendaal, Reitsma, & Bertina, 1996). This polymorphism causes increased CS recognition, increased 3'-end processing, and increased mRNA accumulation and protein synthesis (Danckwardt *et al*., 2004; Gehring *et al*., 2001). A sequence variant located 11 nucleotides downstream of the CS, apparently at a DCPAS, also resulted in increased mRNA expression and protein synthesis, and increased efficiency of the 3'-end formation. Both the fore mentioned variants resulted in gain-of-function, but loss-of-function mutations may conceivably occur at the same functional elements. In fact, J.-M. Chen, Férec, and Cooper (2006a) argued that, considering the genetic and clinical data of the patient, two single nucleotide substitutions in *cis*, with one of the two variants occurring at the nucleotide at position -2 relative to the CS, might lead to a moderate decrease in the efficiency of CS recognition. In this study, the sequences of the three UCPASs of *GJB2* gene, and the two nucleotides immediately preceding the six inferred CSs (see Chapter 6, Annex fig. 1), were invariant in patients and controls.

The 3'UTR of *GJB2* gene contains stretches of four or more T residues localised no more than 30 nucleotides downstream of each of the six inferred CS (see Chapter 6, Annex fig. 1). Several lines of evidence suggest that pentamers consisting of four U residues and any other residue within this pentamer may serve as DCPAS. Pentamers of U residues and A or C single point substitutions in any position of the pentamer were able to mediate polyadenylation, being the U pentamer the most efficient element (Chou, Chen, & Wilusz, 1994). Moreover, stretches of four U residues were able to interact with the 64K subunit of the CstF (MacDonald, Wilusz, & Shenk, 1994). Consistent with these experimental findings, pentamers consisting of four U residues and any other residue within this pentamer, located within 30/32 bases downstream of the CS, have been found to occur in the majority of mammalian (including human) polyadenylation signals containing AAUAAA/AUUAAA UPCAS (F. Chen, MacDonald, & Wilusz, 1995; Zarudnaya, Kolomiets, Potyahaylo, & Hovorun, 2003). Thus, the fore mentioned tracts of T residues might well correspond to DCPASs in the *GJB2* pre-mRNA. However, similarly to what was observed with the UCPASs, the sequences of

these potential DCPASs (see Chapter 6, Annex fig. 1) were invariant in our patients and controls.

As regards the entire 3'UTR of *GJB2* gene, we have found two novel variants in normal-hearing individuals (see sequencing traces in Chapter 6, Annex fig. 2), but no novel mutation was identified in the patients. Noteworthy, we did observe a statistically very significant association of two SNPs (which are in strong LD) with HL. This association could be due to a pathogenic effect of either or both SNPs, or it could be due to an unidentified pathogenic variant in linkage with the two SNPs. We next assessed the possible structural implications of both SNPs, by using the mRNA folding prediction program Mfold. Only the SNP c.*168A>G, which was also found in homozygosity in one of the patients, was predicted to alter the mRNA folding. However, the mRNA folding *in silico* predictions serve merely as an indication and must be considered with caution. Upon conservation analysis we noticed moderately conserved regions in the 3'UTR of *GJB2*, by using the ENCODE database (Birney *et al*., 2007). Both SNPs are localised outside of these regions (see Chapter 6, Annex fig. 3). Nonetheless, any of the two SNPs might be functionally relevant in our species, and it would be interesting to assess, in other populations, their association with HL. We have also observed an increased frequency, in the patient group, of one haplotype comprising four SNPs (CCAC) but, due to the low number of subjects, no statistically valid conclusion could be drawn from our data. The possible association of this haplotype with HL needs to be assessed in a larger sample.

3. Functional assessment of three novel pathogenic *GJB2* mutations

We investigated the role in hearing impairment of three novel *GJB2* mutations, all of which had been identified in Portuguese NSSHL patients.

These mutations, c.-259C>T, c.250G>A (p.Val84Met) and c.487A>C (p.Met163Leu), were identified in two nuclear Portuguese families, presenting with NSSHL (Matos *et al*., 2008, 2007). The pattern of transmission was compatible either with autosomal dominant or mitochondrial inheritance. The presence of the A1555G and A7445G mtDNA mutations, associated with HL, and occurring in several populations, was excluded in the two families. Screening of the *GJB2* coding region in the members of these families revealed the

segregation of either one of the two coding mutations with the HL, which further suggested an autosomal dominant mode of inheritance of the condition. However, Pandya *et al*. (2003) also reported one profoundly hearing-impaired 17-year-old female heterozygous for c.250G>A (p.Val84Met) born to normal-hearing parents. Furthermore, Cheng *et al*. (2005) described one family with apparently recessive HL, in which the affected members presented with profound HL and were compound heterozygous for p.Val84Met (the mutation at the DNA level was not indicated) and either c.35delG (grandmother) or p.Met34Thr (two of the siblings). These genetic data suggested a recessive nature for p.Val84Met. Effectively, we found, in the profoundly hearing-impaired proband, a second *GJB2* mutation (c.-259C>T) in the basal promoter, in *trans* with c.250G>A (p.Val84Met). The basal promoter mutation, not present in the mother, was also identified in heterozygosity in the proband's normal-hearing sister.

Since the c.-259C>T and the p.Met163Leu mutations had not been reported in any other study we have performed functional studies to investigate their suspected pathogenicity. Assessment of the functionality of the c.250G>A (p.Val84Met) mutation was also pursued, since the genetic data regarding that mutation was restricted to our study and two other reports (Cheng *et al*., 2005; Pandya *et al*., 2003), from which its pathogenicity could not be unequivocally inferred.

The effects of the two coding mutations, p.Val84Met and p.Met163Leu (localised to M2 and in E2, respectively), in the functionality of Cx26, as assessed by our functional assays, were distinct.

3.1. The p.Val84Met-Cx26 mutant

The **p.Val84Met-Cx26** mutant protein was able to traffic to the cell membrane, forming gap junction-like structures, although apparently displaying an increased retention in the cytoplasm, as compared to the wild-type protein (Chapter 3, supp. fig. 2). However, the intercellular channels formed solely by p.Val84Met-Cx26 were impermeable to both LY and NBN, suggesting loss-of-function of these channels *in vivo*. We have also observed that the p.Val84Met-Cx26 mutant could co-localise with wtCx26 or wtCx30 at gap junctions. This fact suggested that this mutant might form mixed channels with wtCx26 and/or wtCx30 *in vivo*. However, the functionality of such channels may be affected to some degree by the presence of the defective protein. The possibility that this fact, together with other genetic

and/or environmental factors, might be responsible for the moderate HL of the p.Val84Met carrier, mother of the c.-259C>T/p.Val84Met proband, cannot be discarded.

Further considerations regarding the Val84 residue in Cx26 are due. This amino acid residue is conserved across mammals (Chapter 3, Annex fig. 3). In addition, the valine residue at the corresponding position in other mammalian beta-connexins is invariant (Chapter 3, Annex fig. 4). Several mutations affecting this valine residue in human beta-connexin genes, *GJB2* as well as *GJB1* (Cx32) and *GJB3* (Cx31), were identified in individuals presenting with HL or X-linked Charcot-Marie-Tooth (CMTX) disease (Table 1).

Table 1. Non-synonymous mutations affecting the Val84 amino acid residue in human beta-connexins.

Gene	Mutation		Phenotype[a]	Studies
	DNA	Protein		
GJB2 (Cx26)	c.250G>A	p.Val84Met	HL	Matos (2002) Matos *et al*. (2003) Pandya *et al*. (2003)
	c.250G>C	p.Val84Leu	HL	Kelley *et al*. (1998) Zoll *et al*. (2003) Azaiez *et al*. (2004) Tang *et al*. (2006) Putcha *et al*. (2007)
	c.250G>T	p.Val84Leu	HL	Putcha *et al*. (2007)
	c.251T>C	p.Val84Ala	HL	H. J. Park, Hahn, Chun, Park, and Kim (2000) Pandya *et al*. (2003)
GJB1 (Cx32)	c.250G>A	p.Val84Ile	CMTX	Rouger *et al*. (1997)
GJB3 (Cx31)	c.250G>A	p.Val84Ile	HL	J.-J. Yang *et al*. (2010)

a. The condition affecting the individuals harbouring the referred mutations.

CMTX - X-linked Charcot-Marie-Tooth disease; HL - Hearing loss.

The c.250G>C (p.Val84Leu), in *GJB2*, have been found segregating with HL compatible with a recessive pattern of inheritance (Azaiez *et al*., 2004; Kelley *et al*., 1998). Two other *GJB2* mutations, c.250G>T, also leading to p.Val84Leu, and c.251T>C (p.Val84Ala) have been identified in compound heterozygosity with c.35delG (Pandya *et al*., 2003; Putcha *et al*.,

2007). Kenna *et al*. (2001) reported a patient, with profound HL, homozygous for p.Val84Leu (mutation at DNA level was not provided). A p.Val84Ile mutation (c.250G>A) was identified in *GJB1* and *GJB3* genes, in CMTX and HL patients, respectively (Rouger *et al*., 1997; J.-J. Yang *et al*., 2010; Y. Yuan *et al*., 2009). However, the p.Val84Ile (c.250G>A) mutation in *GJB3* is apparently a polymorphism (Y. Yuan *et al*., 2009). Nonetheless, these genetic data suggest that mutations changing the Val84 residue in beta-connexins, even if for other hydrophobic residues, are not always tolerated. Functional characterization of mutations p.Val84Leu (Beltramello *et al*., 2005; Zhang *et al*., 2005) and p.Val84Met (this study) further supported their pathogenicity. The p.Val84Leu-Cx26 channels displayed impaired permeability to PI and IP3 (Beltramello *et al*., 2005; Zhang *et al*., 2005). However, while p.Val84Leu-Cx26 channels displayed ionic coupling and permeability to LY comparable to wild-type channels (Beltramello *et al*., 2005; Bruzzone *et al*., 2003; Zhang *et al*., 2005), the p.Val84Met-Cx26 channels were impermeable to LY and NBN, and presented reduced electrical coupling. The substitution of Val84 for the bulkier leucine or methionine is likely to increase the distance between M2 and M3 (fig. 1).

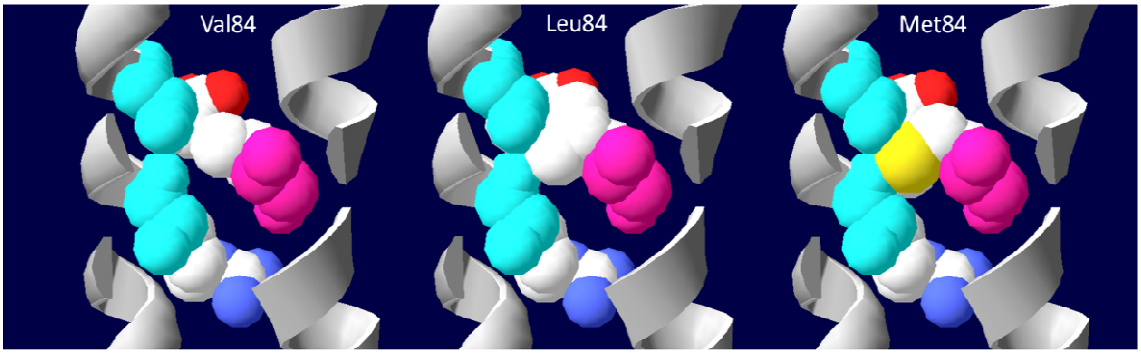


Figure 1. Representation of relative positions of amino acid residues at position 84 of Cx26. The spheres represent van der Waals radii. The main chains of Arg143 and Glu147 are in blue, while the main chain of the amino acid residue at position 84 (Val84, Leu84 or Met84) is in pink. The side chains maintain the color code of Swiss-Pdb Viewer. Replacing Val84 by Leu84 or Met84, in this structure model, led the side chains of both Leu84 and Met84 to contact with the main chains of Arg143 and Glu147. The side chain of Leu84 and Met84 also contact the side chain of Phe146 (not shown, for image clarity). The lowest free-energy rotamers of the amino acid side chains were considered. This simulation was based on the Cx26 model 2zw3 (Maeda *et al*., 2009) and performed using Swiss-Pdb Viewer v4.0.2 (Guex & Peitsch, 1997).

The resulting displacement of M2 might, in turn, might cause displacement of the N-terminus helices, which are thought to form a constriction site (the funnel) at the cytoplasmic entrance of the pore of the connexon, in its open-state (Maeda *et al*., 2009; fig. 2).

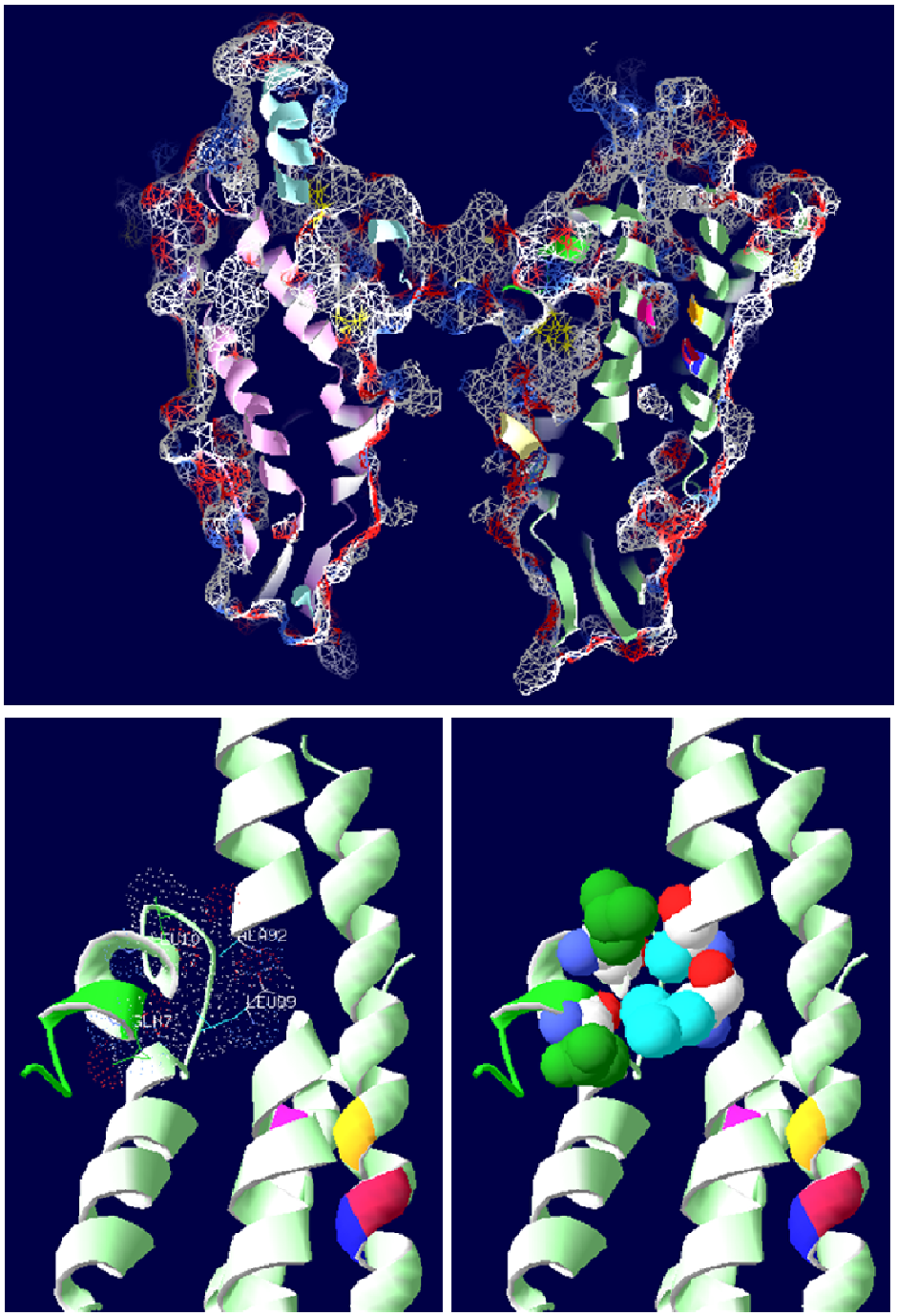


Figure 2. Structure of a Cx26 connexon, showing the funnel structure (upper panel), and representation of the relative positions of amino acid residues Gln7, Leu10 (in N-terminus), Leu89

and Ala92 (in M2), in a Cx26 subunit, showing their close proximity (lower panel). The differently colored ribbons in upper panel represent different connexin subunits within the same connexon. In the lower panel, the side chains of Gln7 and Leu10 are in green and those of Leu89 and Ala92 are in blue, while the backbones of those amino acid residues maintain the color code of Swiss-Pdb Viewer. In the left side image of the lower panel, van der Waals radii are represented as punctate spheres, while in the right side image they are rendered as solid spheres. The protein backbone comprised of amino acid residues 2 to 7 is in bright green, in both panels. The putative displacement of M2, caused by the interaction of leucine or methionine at position 84 (marked in the ribbon in pink) with Arg143 (in orange in the ribbon), Glu147 (in blue in the ribbon) and Phe146 (in red in backbone) (upper and lower panels), likely implies a change in the position of Leu89 and Ala92, the amino acid residues into which the first part of the N-terminus seems to fit, through Gln7 and Leu10 (see lower panel). As a consequence, such displacement might alter the normal position of the N-terminus, and therefore the funnel's diameter. This simulation was based on the Cx26 model 2zw3 (Maeda *et al*., 2009) and performed using Swiss-Pdb Viewer v4.0.2 (Guex & Peitsch, 1997).

As a result, the diameter of the funnel bottom might be reduced, thus preventing the permeation of the fore mentioned tracers or the second messenger IP3. In this hypothesis, the reduced electrical coupling associated with p.Val84Met-Cx26 but not with p.Val84Leu-Cx26 channels, could be due to smaller diameter of the funnel bottom induced by the former mutation, since methionine is bulkier than leucine. Alternatively, the greater deleterious effect of the p.Val84Met mutation on the permeability properties could arise if the displacement of M2 prevented the N-terminus to be in close proximity to the inner wall of the channels, and disrupted the factors stabilizing the funnel structure, preventing the channel to be in the open state. The factors, that promote the stabilization of the funnel structure and the proximity of the N-terminus to the pore wall, could be, as suggested by Maeda *et al*. (2009), the hydrogen bond between Asp2 and Thr5, and the hydrophobic interaction of Met34 and Trp3, of adjacent protomers. In fact, disruption of the latter interaction was hypothesized to release the N terminus from the pore wall, which would then form the occluding "plug" observed within the pore of the Cx26-p.Met34Ala channel (A. Oshima, Tani, Hiroaki, Fujiyoshi, & Sosinsky, 2007, 2008; A. Oshima *et al*., 2011). Therefore, such mechanism might explain the observed impermeability to NBN and LY and the reduced conductance of the p.Val84Met-Cx26 channels.

3.2. The p.Met163Leu-Cx26 mutant

As regards the **p.Met163Leu-Cx26** protein the functional studies performed indicated that the mutant protein presented a trafficking defect, when expressed alone, but could form gap junction-like structures containing both the mutant and wtCx26 or wtCx30. An interesting finding regarding this mutant was that it caused increased cell death. This lethal phenotype was only partially rescued by co-expression of the mutant with either wtCx26 or wtCx30 in a 1:1 ratio, suggesting a dominant-negative effect of the mutant on both wild-type connexins, as regard to cell viability. Co-expression of the mutant with both wild-type connexins in a ratio mimicking a p.Met163Leu heterozygote with two functional *GJB6* (Cx30) alleles, resulted in a cell death rate not statistically different (although slightly higher) from that of cells expressing wtCx26 and wtCx30 (1:1 ratio). Notwithstanding, if the distinct and graded expression of Cx26 and Cx30 reported in the guinea-pig (Zhao & Yu, 2006) also occurs in the human, heterozygosity for p.Met163Leu may explain the HL observed in the patients here reported, which affects the higher frequencies more pronouncedly. Interestingly, heterozygosity for p.Met163Leu has also been described in an Argentinean patient who presented with prelingual, profound NSHL (Dalamón *et al*., 2010). The more severe phenotype of the Argentinean patient might be due to the genetic background that could result in: i) a significantly higher cell death rate induced by the mutation; ii) quantitative differences in the expression of connexin subunits leading to different number of functional channels; iii) a significantly increased susceptibility to the disruption of the gap junction network, each factor of which would strongly compromise the whole cochlea and, thus, all the frequencies. However, a possible progression of the HL could also account for the more severe HL of that patient.

Our results suggest that the increased cell death phenotype associated to p.Met163Leu-Cx26 mutant might not be due to aberrant hemichannel function. By one hand, no gap junctions were observed between cells expressing the mutant protein, and the protein was apparently retained in the cytoplasm, suggesting a trafficking defect. By another hand, high extracellular calcium concentration did not rescue the increased lethality phenotype, as was the case of other dominant Cx26 mutations, p.Gly12Arg, p.Asn14Lys, p.Ala40Val and p.Gly45Glu (Gerido, DeRosa, Richard, & White, 2007; J. R. Lee, Derosa, & White, 2009; Stong, Chang, Ahmad, & Lin, 2006), leading to aberrant hemichannel function in Xenopus oocytes (also in HEK-293 mammalian cells, in the case of p.Gly45Glu). It should

be mentioned that the increased cell death induced by p.Asp50Asn (a Cx26 syndromic skin disease-associated mutation) which could not be rescued by high extracellular calcium concentration in this study, has however been rescued by a similarly high extracellular calcium concentration in one study using Xenopus oocytes (J. R. Lee *et al*., 2009). This discrepancy might be due to intrinsic cellular physiology differences between both species. The amino acid residues Gly12, Asn14 (both localised to the N-terminus), Gly45 and Asp50 (both localised to the E1) of Cx26 are thought to be pore-lining residues (Maeda *et al*., 2009) and therefore potentially involved in voltage-dependent gating. The Ala40 residue, although not contacting the pore lumen, is at the M1/E1 boundary which has been suggested to be involved in voltage sensing, and is adjacent to the Lys41 residue which might have a role in sensing the voltage field (Maeda *et al*., 2009).

Our data suggests that the increased cell death phenotype in human cells induced by p.Asp50Asn and p.Met163Leu might be accounted for a different mechanism, other than aberrant hemichannel gating. Tattersall, Scott, Gray, Zicha, and Kelsell (2009), using human cell lines, provided evidence that endoplasmatic reticulum (ER) stress, leading to the unfolded protein response (UPR), and not “leaky” hemichannels, was the main cause of the increased cell death observed with erythrokeratodermia variabilis (EKV)-associated Cx31 mutants, p.Arg42Pro and Cys86Ser, localised to distinct domains (E1 and T2, respectively). Interestingly, the pattern of cellular localisation of these EKV mutants, which co-localised with upregulated proteasome markers, resembles that of the p.Met163Leu-Cx26, here reported. Thus, it is a possibility that the p.Met163Leu mutation causes increased cell death due to ER-stress. As far as the three dimensional structure of Cx26 is concerned, the p.Met163Leu mutation might lead to clashes between Leu163 and Phe69, and between Leu163 and Gln164. A leucine at position 163 could also tangentially contact Val182 (fig. 3). Indeed, these interactions might affect folding.

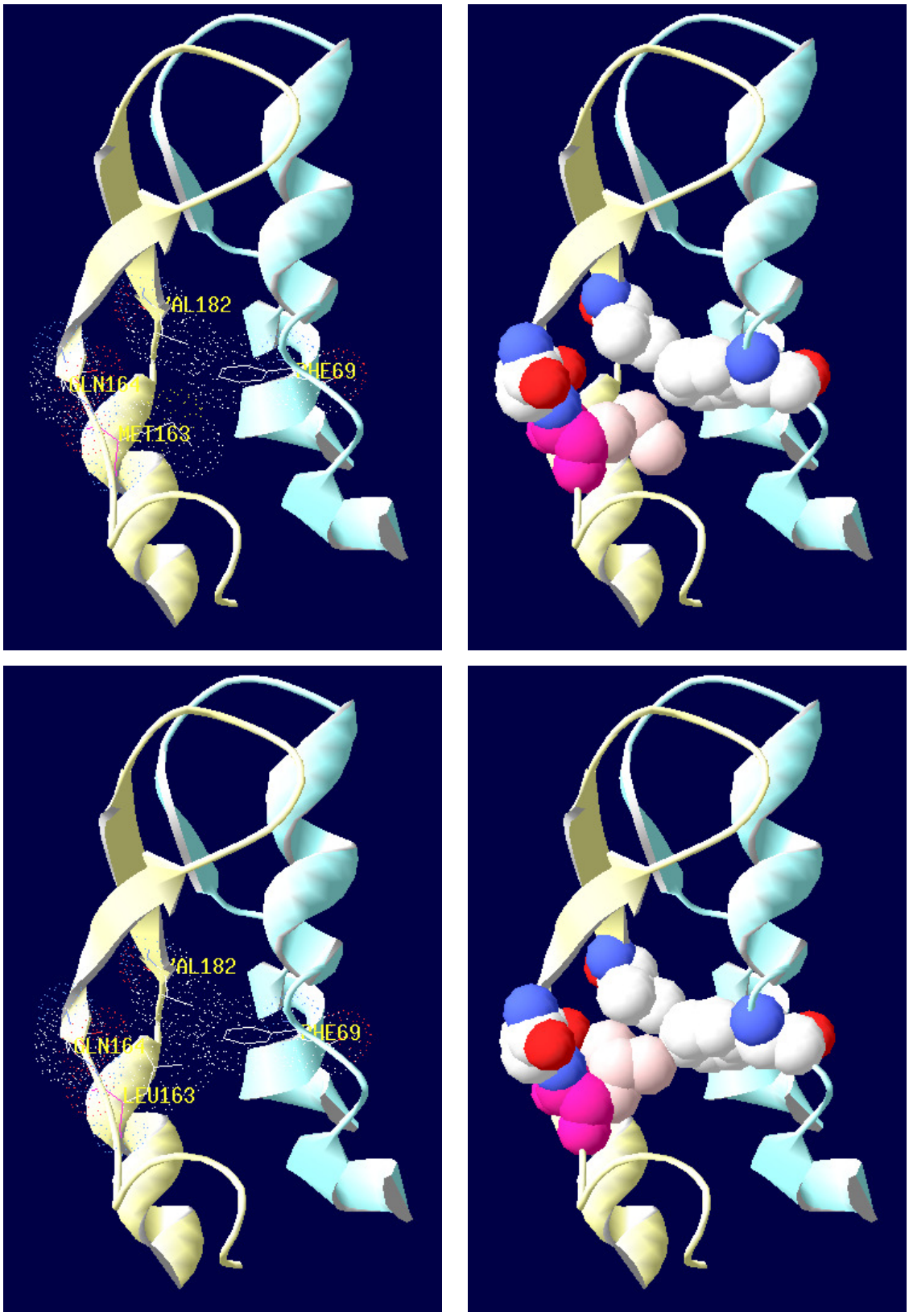


Figure 3. Three dimentional structure of E1 and E2 of Cx26, depicting the methionine or leucine at position 163, and the amino acid residues putatively contacting Leu163. The leucine rotamer here represented is one out of the four possible rotamers (all four rotamers had the same

free-energy value). The backbone of the amino acid residue at position 163 is in dark pink, while the side chain of that residue is in light pink. Atoms of amino acid residues at positions 69, 164 and 182 maintain the color code of Swiss-Pdb Viewer. The blue ribbon represents E1 and the yellow ribbon represents E2. The images in the left represent van der Waals radii as punctate spheres. In the right-hand images van der Waals radii are rendered as solid spheres. This simulation was based on the Cx26 model 2zw3 (Maeda *et al.*, 2009) and performed using Swiss-Pdb Viewer v4.0.2 (Guex & Peitsch, 1997).

3.3. The c.-259C>T mutation

The c.-259C>T mutation changed a GC box, previously proved to be capable of binding to Sp1 and Sp3 transcription factors, and to be critical for *GJB2* basal promoter activity since it disruption by mutation almost abolished reporter gene activity in two human epithelial cell lines (Tu & Kiang, 1998). We have shown that the c.-259C>T mutation greatly impaired promoter activity, in two other human cell lines, HEK-293 and Caco-2. The fact that drastic impairment of basal promoter activity due to mutations in the fore mentioned GC box was consistently observed (four different cell lines), reinforces the notion the Sp1/Sp3-mediated activation of *GJB2* basal promoter is intrinsically dependent on that particular GC box, in spite of the existence of one other GC box, close upstream, also capable of binding to Sp1/Sp3 (Tu & Kiang, 1998). Thus, it is reasonable to assume that the c.-259C>T mutation might impair the basal promoter activity in the supporting cells of the organ of Corti and/or fibrocytes of the spiral ligament and spiral limbus. If this hypothesis is correct, nearly all Cx26 produced in those cochlear regions, in the c.-259C>T/p.Val84Met proband, is expected to be the p.Val84Met-Cx26 mutant form (here shown to impair Cx26 channel function *in vitro*), thereby possibly compromising *in vivo* the function of Cx26 containing intercellular channels. The proband's normal-hearing sister carries only the c.-259C>T mutation, which is also consistent with the functional data. She carries one wild-type *GJB2* allele, which suffices to ensure normal hearing function. Therefore, the analysis of the genetic and the functional data strongly supports that the proband's genotype is most likely the cause of her HL.

Noteworthy, pathogenic mutations affecting important regulatory elements have been identified in 5' untranslated and promoter regions of other connexin genes. A *GJB1* mutation, previously found in a family with CMTX, disrupts an internal ribosome entry site (IRES) within the 5'UTR of the nerve-specific Cx32 mRNA, abolishing translation (Hudder &

Werner, 2000). Two other *GJB1* mutations, also identified in CMTX patients, where shown to cause a reduction in SOX10-dependent activation of the nerve-specific promoter P2 (Bondurand *et al*., 2001; Houlden *et al*., 2004). Osaka *et al*. (2010) found, in a patient with mild Pelizaeus-Merzbacher-like disease, a *GJC2* (Cx47) mutation which completely abolished the SOX10 binding and attenuated the promoter activity.

4. Concluding remarks

The contribution of *GJB2* mutations to NSSHL in Portuguese families has been investigated. As in several other Caucasian populations, c.35delG was the most frequent *GJB2* mutation identified. However, the contribution of *GJB2* mutations to the HL and the relative proportion of c.35delG homozygous cases were not as high as in other Southern European populations.

We have assessed the functionality of two *GJB2* missense mutations and one *GJB2* basal promoter transition mutation, identified in two Portuguese HL families, and for which a pathogenic role was suspected. The results obtained further supported a pathogenic role for each of the three mutations, thus allowing the molecular diagnosis of *GJB2*-related HL for three affected individuals, with benefits to the respective families, since genetic counselling could be provided.

This work also contributed to extend *GJB2* analysis, as regards the role of *GJB2* noncoding regions in HL. We have found the first pathogenic mutation occurring in the basal promoter, allowing for the elucidation of the HL of one affected subject. This finding shows the relevance of searching for mutations beyond the *GJB2* coding region and splice sites, in NSSHL cases, especially in monoallelic individuals. Furthermore, we have found a strong association of two SNPs in the *GJB2* 3'UTR with HL. It would be interesting to investigate whether this association is also present in other populations. Finally, this work provides some relevant annotation regarding known and putative functional elements on the 3'UTR, a region neglected in nearly all molecular diagnosis studies, which might be useful for future investigation.

5. Future Directions

As already mentioned, and it was also shown in this thesis, the *GJB2* gene function may be affected not only by mutations occurring in the coding region, but also by mutations in noncoding regions, such as the promoter and the donor splice site. However, in some NSSHL patients, noncoding mutations may remain to be identified in other noncoding regions of the gene. We have noticed that a ~200 bp stretch within the intron shows some conservation (fig. 4), which suggests it might be important for gene function.

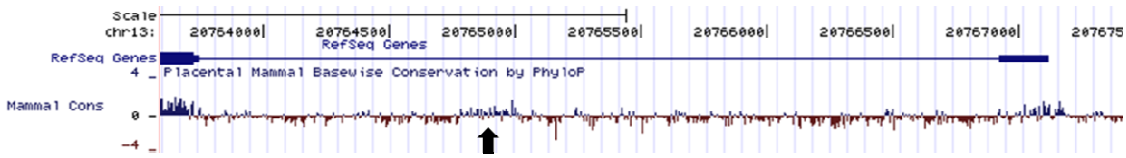


Figure 4. Representation of the conservation of *GJB2* intron among mammals (placental), derived from ENCODE (Birney *et al*., 2007). The intron (thin blue line) contains a well defined region, of ~200 bp, displaying significant overall conservation (black arrow). The right-hand blue box represents exon 1 plus part of the basal promoter. The basal promoter region shows considerable conservation.

As such, pathogenic mutations occurring in that intronic region might exist in some NSSHL cases.

Considering the results here obtained, we propose that screening of *GJB2* basal promoter, exon 1 and donor splice site should be performed in those Portuguese patients harbouring only one pathogenic/controversial *GJB2* coding mutation or one *GJB6* deletion. Since the prevalence of *GJB2* pathogenic noncoding mutations observed in the Portuguese NSSHL cases was low, linkage to DFNB1 should be demonstrated in those patients who harboured no *GJB2* coding mutation and none of the common *GJB6* deletions, as well as in those other patients who harboured only one *GJB2* coding mutation or one *GJB6* deletion, and in whom the screening of the basal promoter, exon 1 and donor splice site had failed to identify further mutations. If there is indication of linkage to DFNB1 then the investigation of *GJB2* sequence should be extended to the other noncoding regions which have been here

investigated, as well as to the fore mentioned ~200 bp stretch of the intron, for which massive parallel sequencing could be a valuable methodology.

REFERENCES

Abe, S., Usami, S., Shinkawa, H., Kelley, P. M., & Kimberling, W. (2000). Prevalent connexin 26 gene (GJB2) mutations in Japanese. *Journal of Medical Genetics*, *37*(1), 41-43.

Azaiez, H., Chamberlin, G. P., Fischer, S. M., Welp, C. L., Prasad, S., Taggart, R. T., del Castillo, I., *et al*. (2004). GJB2: the spectrum of deafness-causing allele variants and their phenotype. *Human Mutation*, *24*(4), 305-311.

Álvarez, A., Del Castillo, I., Villamar, M., Aguirre, L. A., González-Neira, A., López-Nevot, A., Moreno-Pelayo, M. A., *et al*. (2005). High prevalence of the W24X mutation in the gene encoding connexin-26 (GJB2) in Spanish Romani (gypsies) with autosomal recessive non-syndromic hearing loss. *American Journal of Medical Genetics Part A*, *137A*(3), 255-258.

Beltramello, M., Piazza, V., Bukauskas, F. F., Pozzan, T., & Mammano, F. (2005). Impaired permeability to Ins(1,4,5)P3 in a mutant connexin underlies recessive hereditary deafness. *Nature Cell Biology*, *7*(1), 63-69.

Bethesda (MD): National Center for Biotechnology Information National Library of Medicine. (n.d.). Reference SNP(refSNP) Cluster Report: rs9578260. *Database of Single Nucleotide Polymorphisms (dbSNP)*. Retrieved November 7, 2011, from http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=9578260

Bicego, M., Beltramello, M., Melchionda, S., Carella, M., Piazza, V., Zelante, L., Bukauskas, F. F., *et al*. (2006). Pathogenetic role of the deafness-related M34T mutation of Cx26. *Human Molecular Genetics*, *15*(17), 2569-2587.

Birney, E., Stamatoyannopoulos, J. A., Dutta, A., Guigo, R., Gingeras, T. R., Margulies, E. H., Weng, Z. P., *et al*. (2007). Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature*, *447*(7146), 799-816.

Bondurand, N., Girard, M., Pingault, V., Lemort, N., Dubourg, O., & Goossens, M. (2001). Human Connexin 32, a gap junction protein altered in the X-linked form of Charcot-Marie-Tooth disease, is directly regulated by the transcription factor SOX10. *Human Molecular Genetics*, *10*(24), 2783-2795.

Bouwer, S., Angelicheva, D., Chandler, D., Seeman, P., Tournev, I., & Kalaydjieva, L. (2007). Carrier rates of the ancestral Indian W24X mutation in GJB2 in the general Gypsy population and individual subisolates. *Genetic Testing*, *11*(4), 455-458.

Bruzzone, R., Veronesi, V., Gomès, D., Bicego, M., Duval, N., Marlin, S., Petit, C., *et al*. (2003). Loss-of-function and residual channel activity of connexin26 mutations associated with non-syndromic deafness. *FEBS Letters*, *533*(1-3), 79-88.

Cama, E., Melchionda, S., Palladino, T., Carella, M., Santarelli, R., Genovese, E., Benettazzo, F., *et al*. (2009). Hearing loss features in GJB2 biallelic mutations and GJB2/GJB6 digenic inheritance in a large Italian cohort. *International Journal of Audiology*, *48*(1), 12-17.

Chatterjee, S., & Pal, J. K. (2009). Role of 5'- and 3'-untranslated regions of mRNAs in human diseases. *Biology of the Cell*, *101*(5), 251-262.

Chen, F., MacDonald, C. C., & Wilusz, J. (1995). Cleavage site determinants in the mammalian polyadenylation signal. *Nucleic Acids Research*, *23*(14), 2614-2620.

Chen, J.-M., Férec, C., & Cooper, D. N. (2006a). A systematic analysis of disease-associated variants in the 3' regulatory regions of human protein-coding genes I: general principles and overview. *Human Genetics*, *120*(1), 1-21.

Chen, J.-M., Férec, C., & Cooper, D. N. (2006b). A systematic analysis of disease-associated variants in the 3' regulatory regions of human protein-coding genes II: the importance of mRNA secondary structure in assessing the functionality of 3' UTR variants. *Human Genetics*, *120*(3), 301-333.

Cheng, X., Li, L., Brashears, S., Morlet, T., Ng, S. S., Berlin, C., Hood, L., *et al*. (2005). Connexin 26 variants and auditory neuropathy/dys-synchrony among children in schools for the deaf. *American Journal of Medical Genetics Part A*, *139*(1), 13-18.

Chou, Z. F., Chen, F., & Wilusz, J. (1994). Sequence and position requirements for uridylate-rich downstream elements of polyadenylation signals. *Nucleic Acids Research*, *22*(13), 2525-2531.

Cryns, K., Orzan, E., Murgia, A., Huygen, P., Moreno, F., del Castillo, I., Parker, C., *et al*. (2004). A genotype-phenotype correlation for GJB2 (connexin 26) deafness. *Journal of Medical Genetics*, *41*(3), 147-154.

Cucci, R. A., Prasad, S., Kelley, P. M., Green, G. E., Storm, K., Willocx, S., Cohn, E. S., *et al*. (2000). The M34T allele variant of connexin 26. *Genetic Testing*, *4*(4), 335-344.

Dalamón, V., Béhèran, A., Diamante, F., Pallares, N., Diamante, V., & Elgoyhen, A. B. (2005). Prevalence of GJB2 mutations and the del (GJB6-D13S1830) in Argentinean non-syndromic deaf patients. *Hearing Research*, *207*, 43-49.

Dalamón, V., Lotersztein, V., Béhèran, A., Lipovsek, M., Diamante, F., Pallares, N., Francipane, L., *et al*. (2010). GJB2 and GJB6 genes: molecular study and identification of novel GJB2 mutations in the hearing-impaired Argentinean population. *Audiology & Neurotology*, *15*(3), 194-202.

Danckwardt, S., Gehring, N. H., Neu-Yilik, G., Hundsdoerfer, P., Pforsich, M., Frede, U., Hentze, M. W., *et al*. (2004). The prothrombin 3'end formation signal reveals a unique architecture that is sensitive to thrombophilic gain-of-function mutations. *Blood*, *104*(2), 428-435.

D'Andrea, P., Veronesi, V., Bicego, M., Melchionda, S., Zelante, L., Di Iorio, E., Bruzzone, R., *et al*. (2002). Hearing loss: frequency and functional studies of the most common connexin26 alleles. *Biochemical and Biophysical Research Communications*, *296*(3), 685-691.

Dietz, A., Löppönen, T., Valtonen, H., Hyvärinen, A., & Löppönen, H. (2009). Prevalence and etiology of congenital or early acquired hearing impairment in Eastern Finland. *International Journal of Pediatric Otorhinolaryngology*, *73*(10), 1353-1357.

Feldmann, D., Denoyelle, F., Loundon, N., Weil, D., Garabedian, E.-N., Couderc, R., Joannard, A., *et al*. (2004). Clinical evidence of the nonpathogenic nature of the M34T variant in the connexin 26 gene. *European Journal of Human Genetics*, *12*(4), 279-284.

Gehring, N. H., Frede, U., Neu-Yilik, G., Hundsdoerfer, P., Vetter, B., Hentze, M. W., & Kulozik, A. E. (2001). Increased efficiency of mRNA 3' end formation: a new genetic mechanism contributing to hereditary thrombophilia. *Nature Genetics*, *28*(4), 389-392.

Gerido, D. A., DeRosa, A. M., Richard, G., & White, T. W. (2007). Aberrant hemichannel properties of Cx26 mutations causing skin disease and deafness. *American Journal of Physiology Cell Physiology*, *293*(1), C337-C345.

Griffith, A. J., Chowdhry, A. A., Kurima, K., Hood, L. J., Keats, B., Berlin, C. I., Morell, R., *et al*. (2000). Autosomal recessive nonsyndromic neurosensory deafness at DFNB1 not associated with the compound-heterozygous GJB2 (connexin 26) genotype M34T/167delT. *The American Journal of Human Genetics*, *67*(3), 745-749.

Guerci, V. I., Grasso, D. L., Morgutti, M., Amoroso, A., D'Andrea, P., Bicego, M., & Gasparini, P. (2007). Connexin 26 gene: Defining the role of the V153I mutation. *Audiological Medicine*, *5*(3), 200-206.

Guex, N., & Peitsch, M. C. (1997). SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis*, *18*(15), 2714-2723.

Han, S.-H., Park, H.-J., Kang, E.-J., Ryu, J.-S., Lee, A., Yang, Y.-H., & Lee, K.-R. (2008). Carrier frequency of GJB2 (connexin-26) mutations causing inherited deafness in the Korean population. *Journal of Human Genetics*, *53*(11-12), 1022-1028.

Hashemzadeh Chaleshtori, M., Farhud, D. D., & Patton, M. A. (2007). Familial and Sporadic GJB2-Related Deafness in Iran: Review of Gene Mutations. *Iranian Journal of Public Health*, *36*(1), 1-14.

Hashemzadeh Chaleshtori, M., Rad, L. H., Dolati, M., Sasanfar, R., Hoseinipour, A., Zohour, M. M., Pourjafari, H., *et al*. (2005). Frequencies of Mutations in the Connexin 26 Gene (GJB2) in Two Populations of Iran (Tehran and Tabriz). *Iranian Journal of Public Health*, *34*(1), 1-7.

Houlden, H., Girard, M., Cockerell, C., Ingram, D., Wood, N. W., Goossens, M., Walker, R. W. H., *et al*. (2004). Connexin 32 promoter P2 mutations: a mechanism of peripheral nerve dysfunction. *Annals of Neurology*, *56*(5), 730-734.

Houseman, M., Ellis, L., Pagnamenta, A., Di, W., Rickard, S., Osborn, A., Dahl, H., *et al*. (2001). Genetic analysis of the connexin-26 M34T variant: identification of genotype M34T/M34T segregating with mild-moderate non-syndromic sensorineural hearing loss. *Journal of Medical Genetics*, *38*(1), 20-25.

Huculak, C., Bruyere, H., Nelson, T. N., Kozak, F. K., & Langlois, S. (2006). V37I connexin 26 allele in patients with sensorineural hearing loss: evidence of its pathogenicity. *American Journal of Medical Genetics Part A*, *140*(22), 2394-2400.

Hudder, A., & Werner, R. (2000). Analysis of a Charcot-Marie-Tooth disease mutation reveals an essential internal ribosome entry site element in the connexin-32 gene. *The Journal of Biological Chemistry*, *275*(44), 34586-34591.

Hwa, H.-L., Ko, T.-M., Hsu, C.-J., Huang, C.-H., Chiang, Y.-L., Oong, J.-L., Chen, C.-C., *et al*. (2003). Mutation spectrum of the connexin 26 (GJB2) gene in Taiwanese patients with prelingual deafness. *Genetics in Medicine*, *5*(3), 161-165.

Janecke, A. R., Hirst-Stadlmann, A., Günther, B., Utermann, B., Müller, T., Löffler, J., Utermann, G., *et al*. (2002). Progressive hearing loss, and recurrent sudden sensorineural hearing loss associated with GJB2 mutations--phenotypic spectrum and frequencies of GJB2 mutations in Austria. *Human Genetics*, *111*(2), 145-153.

Joseph, A. Y., & Rasool, T. J. (2009). High frequency of connexin26 (GJB2) mutations associated with nonsyndromic hearing loss in the population of Kerala, India. *International Journal of Pediatric Otorhinolaryngology*, *73*(3), 437-443.

Kelley, P. M., Harris, D. J., Comer, B. C., Askew, J. W., Fowler, T., Smith, S. D., & Kimberling, W. J. (1998). Novel mutations in the connexin 26 gene (GJB2) that cause autosomal recessive (DFNB1) hearing loss. *The American Journal of Human Genetics*, *62*(4), 792-799.

Kenna, M., Wu, B. L., Cotanche, D. A., Korf, B. R., & Rehm, H. L. (2001). Connexin 26 studies in patients with sensorineural hearing loss. *Archives of Otolaryngology Head & Neck Surgery*, *127*(9), 1037-1042.

Kenneson, A., Van Naarden Braun, K., & Boyle, C. (2002). GJB2 (connexin 26) variants and nonsyndromic sensorineural hearing loss: a HuGE review. *Genetics in Medicine*, *4*(4), 258-274.

Khandelwal, G., Bhalla, S., Khullar, M., & Panda, N. K. (2009). High frequency of heterozygosity in GJB2 mutations among patients with non-syndromic hearing loss. *The Journal of Laryngology & Otology*, *123*(3), 273-277.

Kudo, T., Ikeda, K., Kure, S., Matsubara, Y., Oshima, T., Watanabe, K. I., Kawase, T., *et al*. (2000). Novel mutations in the connexin 26 gene (GJB2) responsible for childhood deafness in the Japanese population. *American Journal of Medical Genetics*, *90*(2), 141-145.

Lee, J. R., Derosa, A. M., & White, T. W. (2009). Connexin mutations causing skin disease and deafness increase hemichannel activity and cell death when expressed in Xenopus oocytes. *The Journal of Investigative Dermatology*, *129*(4), 870-878.

Lerer, I., Sagi, M., Malamud, E., Levi, H., Raas-Rothschild, A., & Abeliovich, D. (2000). Contribution of connexin 26 mutations to nonsyndromic deafness in Ashkenazi patients and the variable phenotypic effect of the mutation 167delT. *American Journal of Medical Genetics*, *95*(1), 53-56.

Löffler, J., Nekahm, D., Hirst-Stadlmann, A., Günther, B., Menzel, H. J., Utermann, G., & Janecke, A. R. (2001). Sensorineural hearing loss and the incidence of Cx26 mutations in Austria. *European Journal of Human Genetics*, *9*(3), 226-230.

MacDonald, C. C., Wilusz, J., & Shenk, T. (1994). The 64-kilodalton subunit of the CstF polyadenylation factor binds to pre-mRNAs downstream of the cleavage site and influences cleavage site location. *Molecular and Cellular Biology*, *14*(10), 6647-6654.

Maeda, S., Nakagawa, S., Suga, M., Yamashita, E., Oshima, A., Fujiyoshi, Y., & Tsukihara, T. (2009). Structure of the connexin 26 gap junction channel at 3.5 A resolution. *Nature*, *458*(7238), 597-602.

Mahdieh, N., Nishimura, C., Ali-Madadi, K., Riazalhosseini, Y., Yazdan, H., Arzhangi, S., Jalalvand, K., *et al*. (2004). The frequency of GJB2 mutations and the Delta (GJB6-D13S1830) deletion as a cause of autosomal recessive non-syndromic deafness in the Kurdish population. *Clinical Genetics*, *65*(6), 506-508.

Maheshwari, M., Vijaya, R., Ghosh, M., Shastri, S., Kabra, M., & Menon, P. S. N. (2003). Screening of families with autosomal recessive non-syndromic hearing impairment (ARNSHI) for mutations in GJB2 gene: Indian scenario. *American Journal of Medical Genetics Part A*, *120A*(2), 180-184.

Marlin, S., Garabédian, E. N., Roger, G., Moatti, L., Matha, N., Lewin, P., Petit, C., *et al*. (2001). Connexin 26 gene mutations in congenitally deaf children: pitfalls for genetic counseling. *Archives of Otolaryngology Head & Neck Surgery*, *127*(8), 927-933.

Matos, T. D. (2002). *Análise Genética de Famílias Portuguesas com Surdez Não-Sindrómica Autossómica Dominante*. Faculty of Science, University of Lisbon.

Matos, T. D., Caria, H., O'Neill, A., Correia, C., & Fialho, G. (2003). V84M mutation in GJB2 apparently related with dominant hearing impairment. *19th International Congress of Genetics*. Melbourne, Australia.

Matos, T. D., Caria, H., Simões-Teixeira, H., Aasen, T., Dias, O., Andrea, M., Kelsell, D. P., *et al*. (2008). A novel M163L mutation in connexin 26 causing cell death and associated with autosomal dominant hearing loss. *Hearing Research*, *240*(1-2), 87-92.

Matos, T. D., Caria, H., Simões-Teixeira, H., Aasen, T., Nickel, R., Jagger, D. J., O'Neill, A., *et al*. (2007). A novel hearing-loss-related mutation occurring in the GJB2 basal promoter. *Journal of Medical Genetics*, *44*(11), 721-725.

Meşe, G., Londin, E., Mui, R., Brink, P. R., & White, T. W. (2004). Altered gating properties of functional Cx26 mutants associated with recessive non-syndromic hearing loss. *Human Genetics*, *115*(3), 191-199.

Minárik, G., Ferák, V., Feráková, E., Ficek, A., Poláková, H., & Kádasi, L. (2003). High frequency of GJB2 mutation W24X among Slovak Romany (Gypsy) patients with non-syndromic hearing loss (NSHL). *General Physiology and Biophysics*, *22*(4), 549-556.

Morell, R., Kim, H. J., Hood, L. J., Goforth, L., Friderici, K., Fisher, R., Van Camp, G., *et al*. (1998). Mutations in the connexin 26 gene (GJB2) among Ashkenazi Jews with nonsyndromic recessive deafness. *The New England Journal of Medicine*, *339*(21), 1500-1505.

Murgia, A., Orzan, E., Polli, R., Martella, M., Vinanzi, C., Leonardi, E., Arslan, E., *et al*. (1999). Cx26 deafness: mutation analysis and clinical variability. *Journal of Medical Genetics*, *36*(11), 829-832.

Ohtsuka, A., Yuge, I., Kimura, S., Namba, A., Abe, S., Van Laer, L., Van Camp, G., *et al*. (2003). GJB2 deafness gene shows a specific spectrum of mutations in Japan, including a frequent founder mutation. *Human Genetics*, *112*(4), 329-333.

Osaka, H., Hamanoue, H., Yamamoto, R., Nezu, A., Sasaki, M., Saitsu, H., Kurosawa, K., *et al*. (2010). Disrupted SOX10 regulation of GJC2 transcription causes Pelizaeus-Merzbacher-like disease. *Annals of Neurology*, *68*(2), 250-254.

Oshima, A., Tani, K., Hiroaki, Y., Fujiyoshi, Y., & Sosinsky, G. E. (2007). Three-dimensional structure of a human connexin26 gap junction channel reveals a plug in the vestibule. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(24), 10034-10039.

Oshima, A., Tani, K., Hiroaki, Y., Fujiyoshi, Y., & Sosinsky, G. E. (2008). Projection structure of a N-terminal deletion mutant of connexin 26 channel with decreased central pore density. *Cell Communication & Adhesion*, *15*(1), 85-93.

Oshima, A., Tani, K., Toloue, M. M., Hiroaki, Y., Smock, A., Inukai, S., Cone, A., *et al*. (2011). Asymmetric configurations and N-terminal rearrangements in connexin26 gap junction channels. *Journal of Molecular Biology*, *405*(3), 724-735.

Padma, G., Ramchander, P. V., Nandur, U. V., & Padma, T. (2009). GJB2 and GJB6 gene mutations found in Indian probands with congenital hearing impairment. *Journal of Genetics*, *88*(3), 267-272.

Pandya, A., Arnos, K. S., Xia, X. J., Welch, K. O., Blanton, S. H., Friedman, T. B., Garcia Sanchez, G., *et al*. (2003). Frequency and distribution of GJB2 (connexin 26) and GJB6 (connexin 30) mutations in a large North American repository of deaf probands. *Genetics in Medicine*, *5*(4), 295-303.

Park, H. J., Hahn, S. H., Chun, Y. M., Park, K., & Kim, H. N. (2000). Connexin26 mutations associated with nonsyndromic hearing loss. *The Laryngoscope*, *110*(9), 1535-1538.

Pollak, A., Skórka, A., Mueller-Malesińska, M., Kostrzewa, G., Kisiel, B., Waligóra, J., Krajewski, P., *et al*. (2007). M34T and V37I mutations in GJB2 associated hearing impairment: evidence for pathogenicity and reduced penetrance. *American Journal of Medical Genetics Part A*, *143A*(21), 2534-2543.

Poort, S. R., Rosendaal, F. R., Reitsma, P. H., & Bertina, R. M. (1996). A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood*, *88*(10), 3698-3703.

Putcha, G. V., Bejjani, B. A., Bleoo, S., Booker, J. K., Carey, J. C., Carson, N., Das, S., *et al*. (2007). A multicenter study of the frequency and distribution of GJB2 and GJB6 mutations in a large North American cohort. *Genetics in Medicine*, *9*(7), 413-426.

RamShankar, M., Girirajan, S., Dagan, O., Ravi, S., Jalvi, R., Rangasayee, R., Avraham, K., *et al*. (2003). Contribution of connexin26 (GJB2) mutations and founder effect to non-syndromic hearing loss in India. *Journal of Medical Genetics*, *40*(5), e68.

Rouger, H., LeGuern, E., Birouk, N., Gouider, R., Tardieu, S., Plassart, E., Gugenheim, M., *et al*. (1997). Charcot-Marie-Tooth disease with intermediate motor nerve conduction velocities: characterization of 14 Cx32 mutations in 35 families. *Human Mutation*, *10*(6), 443-452.

Santos, R. L. P., Aulchenko, Y. S., Huygen, P. L. M., Van Der Donk, K. P., De Wijs, I. J., Kemperman, M. H., Admiraal, R. J. C., *et al*. (2005). Hearing impairment in Dutch patients with connexin 26 (GJB2) and connexin 30 (GJB6) mutations. *International Journal of Pediatric Otorhinolaryngology*, *69*(2), 165-174.

Scott, D. A., Kraft, M. L., Carmi, R., Ramesh, A., Elbedour, K., Yairi, Y., Srisailapathy, C. R., *et al*. (1998). Identification of mutations in the connexin 26 gene that cause autosomal recessive nonsyndromic hearing loss. *Human Mutation*, *11*(5), 387-394.

Seeman, P., & Sakmaryová, I. (2006). High prevalence of the IVS 1 + 1 G to A/GJB2 mutation among Czech hearing impaired patients with monoallelic mutation in the coding region of GJB2. *Clinical Genetics*, *69*(5), 410-413.

Shahin, H., Walsh, T., Sobe, T., Lynch, E., King, M.-C., Avraham, K. B., & Kanaan, M. (2002). Genetics of congenital deafness in the Palestinian population: multiple connexin 26 alleles with shared origins in the Middle East. *Human Genetics*, *110*(3), 284-289.

Sheets, M. D., Ogg, S. C., & Wickens, M. P. (1990). Point mutations in AAUAAA and the poly (A) addition site: effects on the accuracy and efficiency of cleavage and polyadenylation in vitro. *Nucleic Acids Research*, *18*(19), 5799-5805.

Sirmaci, A., Akcayoz-Duman, D., & Tekin, M. (2006). The c.IVS1+1G>A mutation in the GJB2 gene is prevalent and large deletions involving the GJB6 gene are not present in the Turkish population. *Journal of Genetics*, *85*(3), 213-216.

Skerrett, I. M., Di, W. L., Kasperek, E. M., Kelsell, D. P., & Nicholson, B. J. (2004). Aberrant gating, but a normal expression pattern, underlies the recessive phenotype of the deafness mutant Connexin26M34T. *The FASEB Journal*, *18*(7), 860-862.

Snoeckx, R. L., Hassan, D. M., Kamal, N. M., Van Den Bogaert, K., & Van Camp, G. (2005). Mutation analysis of the GJB2 (connexin 26) gene in Egypt. *Human Mutation*, *26*(1), 60-61.

Snoeckx, R. L., Huygen, P. L. M., Feldmann, D., Marlin, S., Denoyelle, F., Waligora, J., Mueller-Malesinska, M., *et al*. (2005). GJB2 mutations and degree of hearing loss: a multicenter study. *The American Journal of Human Genetics*, *77*(6), 945-957.

Sobe, T., Erlich, P., Berry, A., Korostichevsky, M., Vreugde, S., Avraham, K. B., Bonné-Tamir, B., *et al*. (1999). High frequency of the deafness-associated 167delT mutation in the connexin 26 (GJB2) gene in Israeli Ashkenazim. *American Journal of Medical Genetics*, *86*(5), 499-500.

Stong, B., Chang, Q., Ahmad, S., & Lin, X. (2006). A novel mechanism for connexin 26 mutation linked deafness: cell death caused by leaky gap junction hemichannels. *The Laryngoscope*, *116*(12), 2205-2210.

Tang, H.-Y., Fang, P., Ward, P. A., Schmitt, E., Darilek, S., Manolidis, S., Oghalai, J. S., *et al*. (2006). DNA sequence analysis of GJB2, encoding connexin 26: observations from a population of hearing impaired cases and variable carrier rates, complex genotypes, and ethnic stratification of alleles among controls. *American Journal of Medical Genetics Part A*, *140*(22), 2401-2415.

Tattersall, D., Scott, C. A., Gray, C., Zicha, D., & Kelsell, D. P. (2009). EKV mutant connexin 31 associated cell death is mediated by ER stress. *Human Molecular Genetics*, *18*(24), 4734-4745.

Teek, R., Kruustük, K., Zordania, R., Joost, K., Reimand, T., Möls, T., Oitmaa, E., *et al*. (2010). Prevalence of c.35delG and p.M34T mutations in the GJB2 gene in Estonia. *International Journal of Pediatric Otorhinolaryngology*, *74*(9), 1007-1012.

Thönnissen, E., Rabionet, R., Arbonès, M. L., Estivill, X., Willecke, K., & Ott, T. (2002). Human connexin26 (GJB2) deafness mutations affect the function of gap junction channels at different levels of protein expression. *Human Genetics*, *111*(2), 190-197.

Tu, Z. J., & Kiang, D. T. (1998). Mapping and characterization of the basal promoter of the human connexin26 gene. *Biochimica et Biophysica Acta*, *1443*(1-2), 169-181.

Tóth, T., Kupka, S., Haack, B., Fazakas, F., Muszbek, L., Blin, N., Pfister, M., *et al*. (2007). Coincidence of mutations in different connexin genes in Hungarian patients. *International Journal of Molecular Medicine*, *20*(3), 315-321.

Wattanasirichaigoon, D., Limwongse, C., Jariengprasert, C., Yenchitsomanus, P. T., Tocharoenthanaphol, C., Thongnoppakhun, W., Thawil, C., *et al*. (2004). High prevalence of V37I genetic variant in the

connexin-26 (GJB2) gene among non-syndromic hearing-impaired and control Thai individuals. *Clinical Genetics*, *66*(5), 452-460.

Wilch, E., Zhu, M., Burkhart, K. B., Regier, M., Elfenbein, J. L., Fisher, R. A., & Friderici, K. H. (2006). Expression of GJB2 and GJB6 is reduced in a novel DFNB1 allele. *The American Journal of Human Genetics*, *79*(1), 174-179.

Wu, B.-L., Lindeman, N., Lip, V., Adams, A., Amato, R. S., Cox, G., Irons, M., *et al*. (2002). Effectiveness of sequencing connexin 26 (GJB2) in cases of familial or sporadic childhood deafness referred for molecular diagnostic testing. *Genetics in Medicine*, *4*(4), 279-288.

Yang, J.-J., Wang, W.-H., Lin, Y.-C., Weng, H.-H., Yang, J.-T., Hwang, C.-F., Wu, C.-M., *et al*. (2010). Prospective variants screening of connexin genes in children with hearing impairment: genotype/phenotype correlation. *Human Genetics*, *128*(3), 303-313.

Yuan, Y., You, Y., Huang, D., Cui, J., Wang, Y., Wang, Q., Yu, F., *et al*. (2009). Comprehensive molecular etiology analysis of nonsyndromic hearing impairment from typical areas in China. *Journal of Translational Medicine*, *7*, 79.

Zarudnaya, M. I., Kolomiets, I. M., Potyahaylo, A. L., & Hovorun, D. M. (2003). Downstream elements of mammalian pre-mRNA polyadenylation signals: primary, secondary and higher-order structures. *Nucleic Acids Research*, *31*(5), 1375-1386.

Zhang, Y., Tang, W., Ahmad, S., Sipp, J. A., Chen, P., & Lin, X. (2005). Gap junction-mediated intercellular biochemical coupling in cochlear supporting cells is required for normal cochlear functions. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(42), 15201-15206.

Zhao, H.-B., & Yu, N. (2006). Distinct and gradient distributions of connexin26 and connexin30 in the cochlear sensory epithelium of guinea pigs. *Journal of Comparative Neurology*, *499*(3), 506-518.

Zoll, B., Petersen, L., Lange, K., Gabriel, P., Kiese-Himmel, C., Rausch, P., Berger, J., *et al*. (2003). Evaluation of Cx26/GJB2 in German hearing impaired persons: mutation spectrum and detection of disequilibrium between M34T (c.101T>C) and -493del10. *Human Mutation*, *21*(1), 98.